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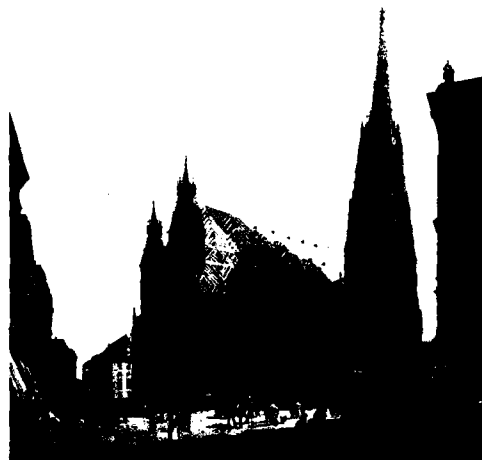
2nd International Symposium on POSITIVE STRAND RNA VIRUSES

June 26 — 30, 1989

Hotel Hilton

Vienna, Austria

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ABSTRACTS

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ABSTRACTS

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S 1

GENOME SIMILARITIES BETWEEN POSITIVE STRAND RNA VIRUSES FROM PLANTS AND ANIMALS: EVOLUTIONARY AND TAXONOMIC IMPLICATIONS
 Rob Goldbach* and Joan Wellink
 Depts. of Virology and Molecular Biology,
 Agricultural University, Wageningen, The Netherlands
 Approx. 75% of the plant viruses studied so far, i.e. at least 470 distinct viruses, have a single-stranded RNA genome of positive polarity. Although these viruses are classified in distinct taxonomic groups and show a wide variation in capsid morphology and genome structure computer-assisted sequence comparisons of the non-structural proteins they encode, have demonstrated that most if not all of them are somehow genetically interrelated. Moreover most plant RNA viruses appear to have (remote) relatives among the animal RNA viruses. Hence the plant comov-, nepo- and polyviruses are genetically related to the picornaviruses, while even a larger number of plant virus groups, a.o. the tobamov-, bromov-, cucumov-, tobamov- and furoviruses, are - to a greater or lesser extent - related to the alphaviruses. Evidence is presented that two major evolutionary mechanisms underlying the genetic relationships reported so far, i.e. divergence from common ancestors and interviral recombination.

S 2

COMPARATIVE AND EVOLUTIONARY ASPECTS OF CORONAVIRAL, ARTERIVIRAL AND TOROVIRAL GENOME STRUCTURE AND EXPRESSION.

Willy Spaan*, Peter Breidenbeek, Ewan Chirside, Twan de Vries, Johan den Boon, Ans Noten, Eric Snijder and Marian Horzinek
 Institute of Virology, Yalelaan 1, 3584 CL Utrecht, the Netherlands.

The genomes of coronaviruses, bovine coronavirus (BEV, the prototype torovirus) and equine arteritis virus (EAV, the prototype arterivirus) are organized into several regions, each containing one or more open reading frames (ORF) which are separated by junction sequences that contain signals for the transcription of multiple 3' co-terminal subgenomic RNAs. Despite the common genome structure and translation strategy, the mode of mRNA transcription is clearly different. In contrast to the coronavirus and EAV mRNAs, BEV mRNAs do not possess a common leader sequence (see abstract E. Snijder). The common leader sequences of coronavirus and EAV mRNAs are the result of a leader primed transcription and splicing, respectively (see abstract T. de Vries).

Significant similarity between the predicted amino acid sequence of a coronavirus and torovirus ORF and the HE1 hemagglutinin of influenza C virus has been identified which could be the result of non-homologous recombination.

A high degree of similarity was also observed in the predicted amino acid sequence of the second ORF of the putative polymerase gene of two coronaviruses belonging to different antigenic clusters (see abstract P. Breidenbeek). Strikingly, several regions of amino acid sequence homology are present in the carboxy terminal part of the coronavirus and torovirus polymerase proteins. On the basis of these data a new superfamily will be proposed.

S 3

THE EVOLUTION OF HUMAN HEPATITIS DELTA VIRUS

John Taylor*, Mei Chao, Mark Yuo, Lania Sharmeen and Sen-Yung Hsien, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

Hepatitis delta virus, HDV, is apparently a subviral agent, in that its transmission always depends upon the presence of an hepadna virus, such as hepatitis B virus, HBV. Recently we have used transfection with cloned DNA to establish that the dependency on HBV is not at the level of genome replication; thus the HBV provides no more than the HDV coat. This dependency together with the fact that the RNA genome of HDV shows no obvious sequence relationship to HBV suggests that we call HDV a satellite of HBV.

The structure and replication of the HDV genome are very similar to known subviral satellites of plants. This analogy even includes the ability of the RNA to self-cleave and to self-ligate. At the same time HDV has special differences relative to the plant agents. Unlike most of the plant agents, HDV encodes a protein: it is a 22 kDa species found both within virions and in the nuclei of infected cells. We have shown that the antigen is essential for successful genome replication but have not yet established its actual role(s). The antigen is apparently not directly involved in RNA-directed RNA synthesis and we are currently testing the hypothesis that it is analogous to the maturases demonstrated for certain group I and II introns. Maybe, HDV can be considered as an infectious intron.

S 4

MOLECULAR GENETIC ANALYSIS OF BROMOVIRUS REPLICATION AND GENE EXPRESSION.

Paul Ahlquist*, Patricia Traylor, Philip Kroner, Rodiya Pacha, and Richard Allison, Institute for Molecular Virology, University of Wisconsin, Madison, WI 53706 USA.

The genomes of brome mosaic virus (BMV) and the related cowpea chlorotic mottle virus (CCMV) are each divided among RNAs 1, 2, and 3 (3.2, 2.9, and 2.1 kb). RNAs 1 and 2 encode 104 and 94 kD trans-acting RNA replication factors, which share extensive amino acid similarity with proteins encoded by togaviruses and many other viruses. RNA3 encodes a 32 kD protein not required for RNA replication and also serves as template for the subgenomic coat protein mRNA, RNA4. We have constructed biologically active cDNA clones for both BMV and CCMV, and are using these in integrated studies of replication and host interactions. Targeted mutagenesis demonstrates that each of three conserved domains in the 104 and 94 kD proteins is involved in RNA replication. Hybrids exchanging selected gene sequences between BMV and CCMV RNA2 have allowed mapping some characteristics of the 94 kD gene, and suggest that some aspects of template selectivity in RNA replication may map in the 104 kD gene. Deletion analysis of several BMV and CCMV RNAs reveals unexpected complexity and variety in the organization of cis-acting signals directing RNA replication. Recent CCMV results show that recombination rapidly rescues viable virus after co-inoculation with independently disabled mutants. Additional gene exchanges show that the differing BMV and CCMV host specificities are controlled by the 32 kD noncapsid gene and at least one other factor.

REPLICATION, RECOMBINATION AND REPAIR OF BROME MOSAIC VIRUS RNA. Timothy C. Hall¹, A.L.N. Rao, Gregory P. Pogue and Lorea E. Marsh. Biology Department Texas A&M University, College Station, TX 77843-3258, U.S.A.

The three genomic RNAs of brome mosaic virus (BMV) encode four proteins. Proteins 1a and 2a, translated from RNAs 1 and 2, respectively, are vital for replication. Protein 3a is thought to be needed for cell-to-cell transmission. The coat protein is genetically borne on RNA3, but is translated from subgenomic RNA4, generated from an internal promoter on RNA3. The sequence of the 3' 200 nucleotides of each of the BMV RNAs is similar, and has a tRNA-like structure that participates in aminoacylation and nucleotidyl transferase functions as well as serving as the promoter for (-) strand synthesis. The 5' end of each RNA contains sequences similar to the internal control regions (ICR) 1 and 2 of pol III promoters. This sequence similarity has suggested a role for pol III factors in the synthesis of infectious (+) strands, which are produced in some 200 fold excess over the (-) strand.

We have used RNA transcripts of cDNA clones containing site-specific mutations and deletions for *in vitro* studies that have mapped viral promoter and tRNA-like functions at the molecular level. This approach permits discrimination between regions of the tRNA-like 3' structure involved in (-) strand promoter (replicase recognition and initiation), tyrosylation and nucleotidyl transferase functions. The promoter yielding subgenomic RNA4 was found to extend for approximately 62 nucleotides, and contains four functional domains. Deletion of the ICR 1 and 2 motifs from the 5' end of BMV RNA reduces promoter activity, supporting our belief that pol III factors may be involved in (+) strand synthesis.

Studies *in vivo*, using barley (a systemic host) plants or protoplasts, and *Chenopodium hybridum* (a local lesion host) plants confirm the validity of interpretations from the *in vitro* studies. Analysis of infectivity of full-length genomic RNAs bearing the defined mutations has revealed repair of the 3' terminus, demonstrating a telomeric-like function for the 3' structure. Recombination of viral sequences has also been seen, as well as the parameters contributing to a high frequency of occurrence in some situations and absence of recombination in others are being investigated.

SELF-CATALYZED LINKAGE OF POLIOVIRUS TERMINAL PROTEIN VPg TO POLIOVIRUS RNA. G.J. Tobin, D.C. Young, M.S. Oberste, B.J. Morasco, and J.B. Flanagan. Department of Immunology and Medical Microbiology, Univ. of Florida, Gainesville, FL 32610 U.S.A.

We have proposed a template-priming model to explain the mechanisms involved in the initiation of (-) strand RNA synthesis and the linkage of VPg to viral RNA. Key predictions of the template-priming model are as follows: RNA synthesis initiates at the 3' end of the template RNA, and a mechanism exists for linking VPg to (-) strand RNA. In recent studies, we have demonstrated that purified polymerase and host factor can initiate RNA synthesis at the 3' end of polyadenylated RNA templates. In addition, we have shown that VPg-linked (-) strand RNA was formed in a self-catalyzed reaction that required VPg, Mg²⁺, and a poliovirus RNA replication intermediate synthesized *in vitro* on poliovirus RNA. The VPg-linkage reaction did not require the viral polymerase, host factor, or ribonucleoside triphosphates and was specific for product RNA synthesized on poliovirus RNA. The covalent nature of the bond between VPg and the RNA was demonstrated by the isolation of VPg-pUp from VPg-linked RNA and phosphotyrosine from VPg-pUp. A model is proposed in which VPg is covalently linked to a 5' terminal UMP residue in (-) strand RNA as the result of a transesterification reaction which involves nucleophilic attack by the VPg tyrosyl-hydroxyl group on a phosphate in the terminal hairpin joining the template and product RNAs.

REPLICATION OF THE SINDBIS VIRUS GENOME.

James H. Strauss, California Institute of Technology, Pasadena CA 9125 USA.

The functions of conserved nucleotide sequences in the alphavirus genome and of the nonstructural proteins translated from the genome have been examined in a number of ways. Site specific mutations, including both substitutions and deletions, have been introduced into conserved domains of the genome and the effect of these changes on the replication of the virus in both vertebrate cells and mosquito cells examined. Functions of nonstructural proteins in RNA replication have been explored by mapping a number of temperature sensitive mutants in several complementation groups, and from the location of these mutations and their phenotypes, at least some of the functions of these proteins can be deduced. The nonstructural proteinase that cleaves the precursor polyprotein to produce the final protein products has been mapped and found to lie in the C-terminal half of protein nsP2. We hypothesize from limited sequence similarities with papain that the proteinase belongs to this superfamily. The kinetics of cleavage of the polyprotein precursors are unusual and suggest that the uncleaved precursor polyproteins may play roles in RNA replication distinct from those of the final products.

MURINE CORONAVIRUS RNA SYNTHESIS J.L. Leibowitz¹, P.W. Zolnick², K.V. Holmes³, and S.R. Weiss². Univ. Texas Medical School-Houston¹, Univ. Pennsylvania Medical School², and Uniformed Services Univ. of Health Sciences³

Murine coronavirus infected cells synthesize a single species of genomic size negative polarity RNA which serves as template for the synthesis of both genome RNA and multiple subgenomic mRNAs. The subgenomic RNAs are bipartite consisting of leader RNA mapping to the 5' end of the genome, with the body of the mRNAs making up a nested set mapping to the 3' portion of the genome.

Temperature-sensitive mutants with defects in RNA synthesis belong to six different complementation groups. When examined in more detail mutants representing these complementation groups display several different phenotypes. Temperature shift experiments have demonstrated that one mutant is defective in a function required very early in replication. Other mutants are blocked at later stages of replication and can be divided into at least two classes, those that can be induced to accumulate leader RNAs and those that do not.

The mouse hepatitis virus RNA polymerase is thought to be encoded within the 5' 20 kb of the MHV genome. Molecular clones representing portions of the putative polymerase gene(s) have been expressed in *E. coli* utilizing a strategy which does not require knowledge of the sequences being expressed. Antisera have been raised to these expressed portions of the presumed MHV polymerase gene(s) and these antisera verified utilizing a novel strategy. Radioimmunoprecipitation experiments utilizing these sera have identified infected cell proteins which are encoded within this portion of the MHV genome. Indirect immunofluorescent studies have demonstrated different intracellular locations for proteins encoded from different portions of the presumed MHV polymerase region of the genome. This supports the hypothesis that multiple gene functions are encoded within this region of the genome.

IN VITRO MANIPULATION OF PICORNAVIRUS cDNAs TO STUDY VIRAL GENE FUNCTIONS

Cristina Giachetti and Bert L. Semler (*)
Department of Microbiology and Molecular Genetics, University of California, Irvine CA 92717, USA

We generated a number of amino acid replacement mutants in the hydrophobic domain contained in poliovirus type 1 polypeptide 3AB. It has been proposed that this membrane-associated precursor of the virus-linked protein VPg may play a role in poliovirus RNA replication acting as a lipophilic carrier for VPg (Semler et al., Cell 28: 405-412). Among the mutants we isolated, mutant Se1-3AB-310/4 showed a strong temperature-sensitive phenotype: viral growth and viral specific RNA synthesis were dramatically reduced at 39°C. Temperature-shift experiments (from 33°C to 39°C) indicated that the mutant was able to synthesize RNA at a normal rate immediately after the shift-up, but longer incubation at 39°C resulted in loss of the RNA synthesis capability. Viral protein synthesis was normal at 39°C during the first hour after the shift from 33°C, but a clear reduction in the overall amount of viral protein synthesized at 39°C occurred afterwards, most likely as a consequence of the lack of new RNA template synthesis at 39°C. No differences in viral polypeptide processing at 39°C were detected, nor were there differences in the 3AB→3A turnover. The only polypeptide difference observed was an altered mobility during SDS-PAGE of these two mutant viral proteins (3AB and 3A). The normal protein synthesis/processing pattern produced by Se1-3AB-310/4, and the results of the RNA synthesis temperature-shift experiments suggest a primary defect in RNA synthesis (which does not involve RNA elongation) as being responsible for the ts phenotype of this mutant. These data provide evidence for a direct role of polypeptide 3AB in poliovirus RNA synthesis.

NATURAL AND ARTIFICIAL POLIO DI PARTICLES

Akio Namoto*

Department of Microbiology, The Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113 Japan.

Defective-interfering (DI) particles of the Sabin strain of type 1 poliovirus were generated on serial passaging. Characterization of many DI particles indicated that the locations of the deletions were limited within the internal genome region encoding viral capsid proteins and that the deletion sites were clustered in certain areas on the genome. Sequence analysis of a number of cloned cDNAs to the DI genomes revealed that every DI genome retained the correct reading frame for viral protein synthesis. These results strongly suggested that one or several viral non-structural proteins might be cis-acting at least a certain stage in viral replication. This notion was proved by the construction of a number of artificial polio DI RNAs followed by the test to measure their ability of RNA replicons. Computer-aided sequence analysis of natural DI genomes gave an insight into the generation mechanism of polio DI particles that was a new copy-choice model. This model was supported by the analysis of the genome of deletion mutants generated from a viable insertion mutant constructed in vitro.

DI-PARTICLES OF HEPATITIS A VIRUS IN CELL CULTURES AND CLINICAL SPECIMENS;

Stegli, G., Nüesch, J.P.F., de Chastonay, J.
Institute for Hygiene & Med. Microbiology, University of Bern, Friedbühlstr. 51, CH-3010 Bern, Switzerland

Hepatitis A virus (HAV) is a unique picornavirus which tends to establish persistent rather than lytic infection upon replication in most cell culture systems. Viral harvests from such cultures contain particles with defective genomes and the ability to interfere with replication of standard HAV. Characteristically, the genomic defects consist in three internal deletions within the region of the genome coding for structural proteins and span nt's 930-4380 (deletion A), 1200-3820 (B), and 1370-3240 (C). In addition, various truncated RNAs were detected lacking either partially or completely the 3' terminal region which is supposed to code for the viral replicase. Type and predominance of deletions A, B, and C varied with the virus strain, with the type of cell used for propagation, and with the number of consecutive in vitro passages of the virus. The most prominent deletion C accumulated to detectable levels as early as five passages after isolation of HAV in cell culture. Moreover, analysis of HAV-RNA purified from clinical specimens revealed the presence of deletions B and/or C in human feces and viremic blood collected in the course of natural hepatitis A as well as in the liver of an experimentally infected marmoset monkey. Appearance of defective HAV genomes both in vivo and in vitro as well as conservation of deletion endpoints under both natural and experimental conditions of replication of HAV may provide an excellent model system to investigate generation and significance of DIPs in viral disease.

MOLECULAR STUDIES ON SINDBIS VIRUS AND YELLOW FEVER VIRUS

C. Rice*, A. Grakoui, T. Chambers, R. Galler, R. Levis, R. Raju, H. Huang, Dept. of Micro. and Immunol., Washington Univ. Sch. of Med., St. Louis, MO 63110-1093, USA

Some of our recent work on Sindbis virus has included a genetic analysis of the promoter for subgenomic RNA synthesis. An insertion mutation in the putative promoter sequence drastically reduced the level of the subgenomic RNA without altering the start site of the RNA. The cis-acting effect of this mutation was demonstrated by incorporation of either a wild-type or mutant junction region into a defective-interfering (DI) RNA, and examining the relative synthesis of DI-derived subgenomic RNA in vivo in the presence of wild-type helper virus. Independent, early passage revertants of the mutant virus were readily isolated and sixteen were characterized in detail. Although all showed an increase in the level of subgenomic RNA synthesis, sequence analysis of the junction region revealed that they were all pseudorevertants, with only two containing potentially compensating changes in the junction region. An assay developed to identify revertants with second-site changes in trans-acting viral components involved in subgenomic RNA synthesis identified at least two such revertants. Mapping of these and other second-site compensating mutations may provide genetic clues as to which virus-specific protein(s) is responsible for interaction with the conserved junction region to promote subgenomic RNA synthesis.

Our work on yellow fever virus (YF) has focused on the identification of the virus-encoded proteins and proteolytic processing of the viral polyprotein. In addition, a system for regeneration of infectious YF virus from cloned cDNA has been developed which should facilitate studies to determine the role of flavivirus proteins and RNA sequences in virus replication and pathogenesis.

ON THE USE OF FULL LENGTH cDNA COPIES IN THE STUDY OF THE REPLICATION AND PERSISTENCE OF THE DEFECTIVE RNA GENOME OF THE HELPER VIRUS.

T. J. Morris, D. J. Morris, A. L. van Kamen, and A. van Kamen. Dept. of Molecular Biology, Agricultural University Wageningen, The Netherlands, 6500 HA Wageningen, The Netherlands.

Full length cDNA clones of both genomic RNAs (B- and M-RNA) of Cowpea Mosaic Virus (CMV) have been used to introduce mutations in the coding region of different viral proteins and in the non-coding region of B-RNA. The effect of mutations on viral RNA replication was determined in cowpea protoplasts inoculated with transcripts from the mutant clones. In addition, inoculation of cowpea plants with mutant RNA made it possible to analyze the effects of mutations on transport of virus through the plant. Results with transcripts from B cDNA clones with mutations in the 5' non-coding region of B-RNA suggest that a hair-pin structure in this region may have a function in viral RNA replication. M-RNA mutants with deletions in the coding regions of the 50K/45K proteins and the capsid proteins are still replicated in protoplasts but fail to infect cowpea plants indicating that both the 50K/45K proteins and the capsid proteins are required for efficient cell-to-cell transport. M-RNA is transcribed into a polyprotein as a result of initiation of translation at start codons at positions 141 and 513. It was found that the AUG at position 141 is essential for the expression of M-RNA *in vivo*. Moreover both the 50K and 45K proteins are required for a successful infection of cowpea plants.

DEFECTIVE INTERFERING RNAs ASSOCIATED WITH PLANT VIRUS INFECTIONS.

T. J. Morris. Dept. of Plant Pathology, Univ. California, Berkeley, CA 94720, USA.

Defective interfering (DI) RNAs have been identified in association with tomato bushy stunt virus (TBSV), a monopartite, icosahedral plant virus of the Tombusvirus group. These DI RNAs significantly attenuate disease development and interfere with the replication of the parent virus. DI RNAs of about 400 nt that are naturally associated with TBSV isolates have been compared to novel DI RNA species generated *de novo* by high multiplicity passage in several plant hosts. One such DI RNA, derived during ten consecutive high m.o.i. passages, is a 600-nt single-stranded RNA that is efficiently encapsidated in the TBSV capsid protein, replicates to high titre, and protects the host from the normally lethal necrosis associated with helper virus infection. Several independent cDNA clones made to this DI RNA are colinear deletion mutants of the helper virus consisting of sequences from both the 5' and 3' non-coding regions of the viral genome as well as a portion of internal sequence from the viral polymerase domain. This motif is similar to that of some newly identified DI-like RNA species associated with turnip crinkle virus, a distantly related member of the Carmovirus group. We are currently using *in vitro* transcripts of natural and artificially modified DI clones to identify sequences important in the replication, symptom modulation, and encapsidation of these RNAs.

INITIATION OF PROTEIN SYNTHESIS IN PICORNAVIRUS mRNA AND PROTEOLYTIC PROCESSING OF THE VIRAL POLYPROTEIN, S.K.Jang, C.U.T.Hellen, H.G.Krausslich, C.K.Lee, C. Mirzayan, Q. Reuter, C. Holscher and E. Wimmer* Dept. Microb., SUNY at Stony Brook, Stony Brook, NY 11794. The large size of the 5'-nontranslated region, and the absence of a capping group in picornavirus mRNA have led us to carry out experiments aimed at investigating the mechanism of initiation of protein synthesis of these viruses. We will present evidence suggesting that the formation of an initiation complex occurs at an "internal ribosome entry site" (IRES) without the need for a free 5' end. The IRES element is several hundred nucleotides long and, according to the work of V. Agol, highly structured. The polyprotein is myristoylated soon after initiation of synthesis; inhibition of myristoylation by mutagenesis renders viral RNA transcripts non-infectious. Mutagenesis of components of the polyprotein has produced new insight in viral protein function. For example, VPg was found to be involved in steps of viral proliferation following RNA synthesis. Expression of viral proteinase 3C (suggested to be a trypsin-like cysteine proteinase) in *E. coli* has allowed us to produce homogenous enzyme suitable for crystallographic studies. The enzyme cleaves specifically synthetic peptides with kinetics mimicking that observed *in vivo*. Purified 3C, however, cannot cleave efficiently the P1 capsid precursor, although it can process *in vivo* and *in vitro* a Q*G cleavage site engineered into an antigenic site of the virion. Site-directed mutagenesis of 2A cleavage sites suggests that intra- and intermolecular cleavages carried out by 2A differ in their requirements for the cleavage signal.

UNIQUE FEATURES OF INITIATION OF PICORNAVIRUS RNA TRANSLATION.

M.T.Howell, A. Kaminski and R.J.Jackson*. Dept. of Biochemistry, U. of Cambridge, Tennis Court Rd. Cambridge CB2 1QW, U.K.

Recent evidence has led to the suggestion that ribosomes select the correct initiation site on picornavirus RNAs by scanning *not* from the 5'-end but from an internal ribosome entry site located within the 5'-untranslated segment (5'-UTR). We have examined this proposition using cell-free translation of RNAs transcribed *in vitro* from a variety of constructs comprising segments of picornaviral 5'-UTR linked to reporter genes. In the case of encephalomyocarditis virus (EMC) RNA, it was shown that the correct initiation site, the 11th AUG from the 5'-end, is not selected by a mechanism involving scanning from an upstream entry site. The immediate upstream AUG codons, AUG-8 through AUG-10, are completely silent in RNAs which include most of the EMC 5'-UTR sequences downstream of the poly C tract, but are fully functional in constructs with shorter 5'-UTR segments which are translated by a conventional cap-dependent scanning mechanism. With RNAs that include the long 5'-UTR, ribosomes seem to bind directly and exclusively to AUG-11 without scanning the upstream sequences. In contrast to this initiation at a very specific site on constructs with EMC 5'-UTR sequences, our results with constructs which include segments of the poliovirus 5'-UTR suggest that ribosomes initiate preferentially at the first AUG codon downstream of nt. 580-600, regardless of the exact position of this AUG codon. This is consistent with the comparative 5'-UTR sequences of polioviruses and human rhinoviruses, and with the following observations of other groups: (i) deletions of the poliovirus 5'-UTR from about nt. 600 to nt. 730 have little effect on translation efficiency; (ii) the insertion of an AUG-containing sequence into this region is inhibitory to correct expression of the poliovirus coding sequence. These observations could be explained by a model of ribosome binding at an internal entry site, followed by linear scanning of the RNA starting from a position close to nt. 600.

INVESTIGATION OF CAP-INDEPENDENT TRANSLATION OF THE POLIOVIRUS RNA AND THE CELLULAR mRNA ENCODING HEAT SHOCK-LIKE PROTEIN GRP78/BiP.

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Dept. of Biochemistry, Biophysics and Genetics,
University of Colorado HSC, Denver, CO 80262, USA.

Poliovirus interferes with the translational machinery of its host by inactivating the cap-binding protein complex eIF-4F. Subsequently, cellular mRNAs bearing a m7G-5'cap structure can not be translated, while the viral RNA, which does not have a 5'cap, can be used as a functional mRNA by a mechanism termed "cap-independent translation".

Nonetheless, we have discovered that at least one cellular mRNA, encoding the stress-induced protein GRP78/BiP can be translated at a dramatically increased level at a time during poliovirus infection when cap-dependent translation of cellular mRNAs is inhibited. Data are presented which suggest potential roles of heat shock and heat shock-like proteins in poliovirus-infected cells.

It has been postulated that cap-independent translation is mediated by internal binding of the 40S ribosomal subunit to the mRNA. In order to elucidate this mechanism, we have used a novel electrophoretic assay, in which RNA-protein complexes migrate more slowly in polyacrylamide gels, to identify factors which interact with the 5'noncoding region of the viral RNA. We have identified protein factors that bind specifically to certain sequences in the 5'noncoding region of the viral RNA, and we are currently assaying the possible functions of these factors and their cognate sequences in cap-independent translation.

MYRISTYLATION OF POLIOVIRUS VP4 CAPSID PROTEINS

Carol Reynolds, Lisa Curry, Nicola Moscufo, John Simons and Marie Chow*, Dept. of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139 USA

The VP4 capsid proteins of poliovirus is N-terminally modified with myristic acid, the fourteen carbon saturated fatty acid. The modification reaction appears to occur co-translationally with viral protein synthesis and can be catalyzed by host-encoded enzymes. To characterize this modification, mutations have been site-specifically placed in the N-terminal amino acid sequence of the VP4 protein. Substitutions of the N-terminal glycine residue with a wide variety of amino acids prevent this modification from occurring, indicating that a N-terminal glycine is absolutely required for this reaction. Additionally, upon transfection into HeLa cells, these mutant genomes fail to yield infectious virus. Thus, myristate modification appears to be required for virus viability and mutations in the virus that prevent myristoylation of its VP4 proteins are lethal. Amino acid substitutions at other residues in VP4 display altered levels of myristate modification and generate viruses that display attenuated growth phenotypes. The characterization of these viruses will be discussed.

THE SIGNAL SEQUENCE OF THE P62 PROTEIN OF SEMLIKI FOREST VIRUS IS INVOLVED IN INITIATION BUT NOT IN COMPLETING CHAIN TRANSLOCATION

Henrik Garoff*, Danny Huylebroeck, Andrew Robinson, Ulrich Tillman, Dept. of Molecular Biology, Huddinge University Hospital, Karolinska Institute, Sweden

The assembly mechanisms for the Semliki Forest virus (SFV) are based on the existing pathways for membrane assembly in the animal cell. Because SFV has evolved several rather unique modes of using these pathways this virus has proven a valuable model to study membrane assembly in general. For example the question of signal sequence function in chain translocation across the membrane of the endoplasmic reticulum (ER) has recently been addressed using SFV.

So far it has been demonstrated that the signal sequence of proteins which are made at the ER functions both at the level of protein targeting to the ER and in initiation of chain translocation across the ER membrane. However, its possible role in completing the process of chain transfer (c.f. Singer, S.J., P.A. Maher, and M.P. Yaffe, Proc. Natl. Acad. Sci. USA, 1987, 84:1015-1019) has remained elusive. In the present work we show that the p62 protein of SFV contains an uncleaved signal sequence at its N-terminus and that this becomes glycosylated early during synthesis and translocation of the p62 polypeptide. As the glycosylation of the signal sequence most likely occurs after its release from the ER membrane our results suggest that this region has no role in completing the transfer process.

MOLECULAR GENETIC ANALYSIS OF A PLANT VIRUS PROTEINASE AND ITS CLEAVAGE SITE.

T. Dawn Parks, S.M. Cary, and W.G. Dougherty*, Department of Microbiology, Oregon State Univ., Corvallis, OR, USA.

Tobacco etch virus (TEV), a polyvirus, expresses its genetic information as a single high mol wt polyprotein from a genome length RNA. This polyprotein is processed by at least two viral encoded proteolytic activities associated with 87,000 dalton (kDa) and 49kDa proteins. Both proteins autocatalytically release from the polyprotein, while the 49kDa proteinase also cleaves at three other sites. The cleavage site requirements of the 49kDa proteinase have been examined using substrates synthesized in a cell-free transcription/translation system. The TEV 49kDa proteinase recognizes a heptapeptide sequence and cleaves the polyprotein between Gln-Ser and Gln-Gly dipeptides. Within this seven amino acid cleavage sequence, the P6 Glu, the P3 Tyr and the P1 Gln are conserved and are essential in defining a functional cleavage site. Amino acids occupying the P5, P4 and P2 positions vary between natural TEV cleavage sites, and in cell-free assays, affect the cleavage reaction profile. Two TEV cleavage sites have been analyzed in detail; the 50kDa/71kDa and the 58kDa/30kDa TEV product junctions. The 50kDa/71kDa cleavage site is processed "slowly", while the 58kDa/30kDa site is processed "rapidly" in cell-free processing assays. Exchanging the amino acids in the non-conserved positions with those residues found at the other cleavage site changes the reaction profiles. This raises the possibility that TEV may regulate gene expression post-translationally by differential processing of particular cleavage sites.

STRUCTURE AND ASSEMBLY OF POLIOVIRUS

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We have solved the structure of several strains of poliovirus including the neurovirulent type 1 Mahoney strain, the attenuated type 1 Sabia strain, and a mouse adapted type 2 - type 1 chimera. In addition, we have begun to analyze the structure of native, antigenic, dissociable empty capsids from the type 1 Mahoney strain. The implications of these structures for the structural determinants of serotype specificity, host range, stability, and conformation transitions of the virus will be discussed.

THE STRUCTURE OF FOOT-AND-MOUTH DISEASE VIRUS

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The structure of crystals of FMDV (serotype O; BFS 1860) has been determined at 2.9 Å resolution. Refinement against all data (including weak and negative intensities) in the range 5-2.9 Å using XPLOR, has reduced the R-factor to 16% with an RMS deviation in bond lengths from ideal values of 0.017 Å, with no manual rebuilding of the model.

The overall structure of the capsid and the arrangement of the proteins within it are similar in gross terms to that described for other picornaviruses, however, there are a number of unique features. The canyon or pit found in other picornaviruses appears to be absent; this has important consequences for cell attachment. The major immunogenic site (the so-called FMDV loop, residues 140-160 approx.) forms a disordered protrusion. This offers a satisfying structural explanation for the peculiar success of synthetic peptide vaccines against FMDV which might have broad implications for the design of such vaccines. Several lines of evidence from our work and that of others have implicated this FMDV loop in cell attachment and there is evidence that the cellular receptor for FMDV is a member of the integrin family.

We have investigated the structure of the drug binding site identified by Rossmann for the rhinoviruses. These results will be discussed.

ASSEMBLY AND RNA PACKAGING IN SMALL SPHERICAL VIRUSES: RESULTS FROM HIGH RESOLUTION CRYSTALLOGRAPHY

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There are a limited number of nucleoprotein complexes that can be isolated directly from cells and examined using X-ray crystallography. Examples under investigation include the ribosome, the nucleosome core particle and spherical viruses. Only viruses have been examined at a resolution sufficient to reveal atomic detail. Coat protein structures of spherical viruses infecting mammals, insects and plants have been determined at near atomic resolution, however, protein-nucleic acid interactions in these viruses have been only indirectly suggested by disorder in portions of the protein subunits and by the distribution of basic residues on the interior surface of the capsid. We report the structure of a spherical virus particle in which about 20% of the packaged RNA is well ordered. Portions of the ribonucleotide chain bind to specific regions of the viral subunits and display the symmetry of the capsid protein. The RNA is packaged in units of roughly 33 ribonucleotides that circulate about the trimer axes of the capsid. Analysis of the protein subunits shows that there is a striking similarity between picornavirus capsids and the beanpod mottle virus (BPMV) protein shell, thus supporting the relationship between picornaviruses and the plant comoviruses already suggested.

THE CORE PROTEIN OF THE ALPHAVIRUS AND FLAVIVIRUS GENOMES: AN IMPORTANT PART IN THE REPLICATION OF GENOMES AND THE ASSEMBLY OF VIRUS PARTICLES. S. C. JOHNSON, Department of Virology, Harvard University, 665 Huntington Avenue.

Studies on the metabolism of alphavirus core proteins provide insight into the recruitment and disassembly of viral cores in vitro and of the fate of the particles of virus particles early in infection allow to propose the following model for assembly and disassembly of alphaviruses in vivo: During virus synthesis newly synthesized core protein binds to the large ribosomal subunit. Only in the late stages of viral multiplication, when a further saturation of ribosomal binding sites has been reached, is the core protein efficiently used for the assembly of cores. The ribosomal C protein binding sites, recognized by the core of the assembling virus, are then, bind the core protein efficiently, and thereby disassemble the core.

Sequence analyses of the core protein of the West Nile Flavivirus show that the newly synthesized C protein contains a hydrophobic carboxy-terminal sequence which probably functions as a membrane anchor. Core protein present in virus particles does not contain this segment. Removal of the hydrophobic sequence might convert a membrane-associated complex of genome RNA and core protein which assembles into virus into a core which early in infection can be released from the viral envelope and then liberates the genome for translation.

EVIDENCE FOR SPECIFICITY IN THE ENCAPSIDATION OF SINDBIS RNAs

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In previous studies we had identified sequences in DI RNAs of Sindbis virus that are essential for replication and packaging, but could not distinguish between those two steps. We have now identified a region of the viral genome that confers specificity in the binding of RNA to the capsid protein *in vitro*. This region which extends from nucleotide 683 to 1255 in virion RNA is also present in DI RNAs. A DI RNA that lacks these sequences replicates in infected cells, but is not packaged, providing evidence that this domain plays a role in encapsidation *in vivo*. In the course of these and other studies on packaging, we discovered that Sindbis RNAs can undergo recombination. These results will be discussed.

THE CELL BIOLOGY OF A TROJAN HORSE: EARLY EVENTS IN ALPHAVIRUS INFECTION. A. Helenius*, Susan Froehner, Ila Singh, Sandra Schmid and Ira Mellman. Dept. Cell Biol., Yale School of Med., New Haven, Ct, USA.

Alphaviruses enter their host cells by receptor-mediated endocytosis followed by acid triggered fusion in endosomal vacuoles. By studying the uptake and the acid-induced conformational changes in the spike glycoproteins in normal and acidification defective CHO cells, we have demonstrated that the organelle of entry is the early endosome. SFV does not penetrate from primary endocytic vesicle, nor normally from late endosomes or lysosomes. Our evidence suggests, moreover, that replication of viral RNA and synthesis of nonstructural as well as of structural proteins occurs in structures associated with the cytoplasmic surface of cytopathic vacuoles (which we find to be modified secondary lysosomes). The lysosome associated material contains nsP3 and nsP4, and probably constitutes a "factory" for synthesis and assembly of nucleocapsids. The uncoating of the viral nucleocapsids remains the least understood step in the pathway. We find that the low pH in endosomes is not required for uncoating of incoming nucleocapsids: capsids isolated from virus are infectious when microinjected into cell. (NIH R37 AI18599).

CHARACTERIZATION AND EXPRESSION OF THE RECEPTOR GLYCOPROTEIN FOR MOUSE HEPATITIS VIRUS (MHV) IN SUSCEPTIBLE CELLS

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Mouse hepatitis virus (MHV) is a coronavirus which infects liver, intestine, lung, respiratory epithelium, and spinal cells of susceptible strains of mice. Virus binds to purified liver and intestinal brush border membranes from susceptible B6H mice but not to membranes from resistant H-2 transgenic mice (J. Virol. 61:295, 1987). The receptor is a 100 to 110 k glycoprotein, and the E2 glycoprotein of MHV-A59 appears to bind to a linear amino acid sequence of the receptor. A monoclonal anti-receptor antibody (MAb) blocks binding of virus to receptor and prevents infection *in vitro*. The epitope of the receptor which binds virus and blocking MAb is not expressed on cells of other species. Thus, the limited host range of MHV is probably due to the specificity of the virus-receptor interaction. The same receptor glycoprotein appears to be required for binding of MHV-A59 to all susceptible cell types. Characterization of the receptor glycoprotein and cloning of the receptor gene are now in progress.

MOLECULAR CHARACTERIZATION OF THE MAJOR GROUP RHINOVIRUS RECEPTOR REVEALS IDENTITY TO ICAM-1. Joanne E. Tomassini, Donald Graham, Corvill M. Dewitt, Donald W. Lineberger, and Richard J. Colonna*. Dept. of Virus & Cell Biology, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486

A 90 kDa surface glycoprotein was previously isolated and shown to be required for infection by the "major" group of Human Rhinovirus (HRV) serotypes (J. Virol. 58:290-295). Amino acid sequence of the receptor protein was obtained from isolated CNBr and tryptic peptides. Using degenerate oligonucleotides predicted from the peptide sequences, four cDNA clones were identified that encode a 3 kb mRNA. The clones were ligated together, subcloned into a SV40 expression vector, and used to transfect receptor negative Vero cells. Results showed that transfected cells expressed receptor molecules capable of binding HRV and a monoclonal antibody which recognizes the "major" group HRV receptor. The cloned receptor gene displayed nearly identical sequence homology to the intercellular adhesion molecule 1 (ICAM-1) and indicates that the two surface proteins are one and the same. Both proteins have identical mass, carbohydrate composition, and tissue distribution. In addition, "major" group receptors on HeLa cells could be induced with various cytokines in a manner similar to the ICAM-1 ligand. A similar induction of the HRV "minor" group receptor was not observed. Molecular studies were initiated to map the virus and antibody sites on the ICAM-1 ligand.

IDENTIFICATION OF THE MAJOR HUMAN RHINOVIRUS RECEPTOR. Allen McClelland* and Jeffrey M. Greve. Molecular Therapeutics Inc., West Haven, CT USA.

The major human rhinovirus receptor gene has been introduced into mouse L cells by transfection of human genomic DNA and FACS selection using both virus binding and an anti-receptor monoclonal antibody. Purified 120 kd receptor protein isolated from transfectants binds rhinoviruses of the major receptor group in vitro. Protein sequence of 105 residues of the receptor showed 100 % identity with ICAM-1. The DNA sequence of a full length receptor cDNA clone isolated from transfectants confirmed that the major human rhinovirus receptor and ICAM-1 are the same molecule. Experiments to identify virus binding determinants of this receptor and to produce soluble inhibitors of viral attachment will be described.

INHIBITION OF CYTOPATHOGENICITY INDUCED BY PICORNAVIRUSES BY A MONOCLONAL ANTIBODY TO INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1). Vincent J. Merluzzi, Ph.D.* and Robert Rothlein, Ph.D., Boehringer Ingelheim Pharmaceuticals, Inc. 90 East Ridge Road, Box 368, Ridgefield, CT 06877. Recently, antibodies directed toward Intercellular Adhesion Molecule-1 (ICAM-1) have been shown to inhibit the attachment of the major subgroup of rhinoviruses to their receptor. The receptor for this subgroup has been subsequently shown to be ICAM-1.

The effect of monoclonal antibodies to adhesion proteins were tested on picornavirus-induced cytopathogenic effect (CPE) assays in vitro. Several rhinovirus strains representing the major and minor receptor subgroups as well as three coxsackie strains, one polio strain and two non-picornavirus strains [Herpes Simplex 1 (HSV-1) and Influenza A] were studied using HeLa cells as targets. Monoclonal antibodies to ICAM-1 inhibited CPE induced by picornaviruses that bind to the major rhinovirus receptor subgroup. These same antibodies had no effect on picornaviruses that attach to the minor rhinovirus receptor subgroup or unrelated virus receptors. Antibodies to Lymphocyte Function-Associated Antigen-1 (LFA-1), the natural binding partner for ICAM-1, did not inhibit CPE induced by rhinoviruses. Cytopathogenicity induced by HRV54 (major subgroup) on the JY lymphoblastoid cell line was inhibited by antibodies to ICAM-1. In addition, HRV54 induced capping of ICAM-1 on JY cells as detected by immunofluorescence. This observation suggests a possible mechanism by which HRV54 enters target cells.

IDENTIFICATION OF A SECOND CELLULAR RECEPTOR FOR A COXSACKIEVIRUS CB3 VARIANT (CB3-RD). K-H.L. Hsu, S. Paylini, B. Alstein, and R.L. Crowell. Hahnemann Univ. Sch. of Med., Philadelphia, PA 19102.

Coxsackievirus B3-RD (CB3-RD) is a host range virus variant which will grow in human RD cells. It recognizes both a new receptor (HR2) on RD cells and the receptor (HR1) for parental CB3 on HeLa cells (Hsu et al J.Virol. 62:1647, 1988). CB3-RD also binds to a saturable receptor (HR3) on human erythrocytes which is specifically inhibited by a monoclonal antibody RmCA, which also recognizes HR2 on both HeLa and RD cells. HeLa, RD and erythrocyte cell membranes were solubilized with detergents. Virus or antibody binding activity was determined in a filter binding assay (Bio-dot assay) using ³⁵S-labeled CB3-RD or ¹²⁵I-labeled RmCA as probes. The binding sites on all three cells for either probe were sensitive to 2-mercaptoethanol treatment, but insensitive to trypsin. Solubilized cell membrane proteins were separated by non-reducing SDS-polyacrylamide gel electrophoresis, blotted on nitro-cellulose membranes and probed with either ³⁵S-CB3-RD or ¹²⁵I-RmCA. A 60 kd band was identified in HeLa cell (also had a 55 kd band), RD cell and erythrocyte membranes which was distinct from the 50 kd receptor protein described previously for the HeLa cell HR1. Supported by NIH Grant No AI03771

MOLECULAR GENETICS OF CELLULAR RECEPTORS FOR POLIOVIRUS

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The first event in poliovirus replication is attachment to a cell receptor. To identify this receptor and determine its role in viral tissue tropism, cDNA clones encoding functional poliovirus receptors were isolated. The cDNA clones encode transmembrane polypeptides that are new members of the immunoglobulin superfamily. Northern hybridization analysis indicates that poliovirus receptor transcripts are expressed in a wide range of human tissues, in contrast to the limited expression of virus binding sites, which suggests that additional factors or modifications of the receptor protein are required to permit poliovirus attachment. To identify the site of poliovirus attachment on the cell receptor, deletions of receptor cDNAs were constructed which will be assayed for the ability to support poliovirus binding and infection. To determine the structure of the receptor at the cell surface, trpE-poliovirus receptor fusion polypeptides have been synthesized and used for production of rabbit polyclonal anti-receptor antiserum. To elucidate the basis for the ability of the P2/Lansing strain of poliovirus to infect mice, murine genomic and cDNA clones were isolated that are homologous to human poliovirus receptor cDNA. Northern analysis indicates that the murine homologue of the polio virus receptor is expressed in many mouse tissues. Experiments are under way to determine whether the murine receptor homolog encodes poliovirus binding sites.

THE ANTIGENIC STRUCTURE OF FOOT AND MOUTH DISEASE VIRUS AND ITS RELEVANCE TO SYNTHETIC VACCINES

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The structure of foot and mouth disease virus (FMDV) has been solved at a resolution of 2.9 Å by X-ray diffraction techniques. The overall structural organisation of the particle is similar to that seen in other picornaviruses but there are several unique features. Many of these help to explain its characteristic physical and biological properties. In particular the canyon or pit found at the surface of other picornaviruses is lacking, which has important implications for cell attachment and the process of infection. Also there are 60 large disordered protrusions at the surface corresponding to the major antigenic site. This disorder is of particular interest in relation to the striking ability of linear synthetic peptides to induce protective immunity against foot and mouth disease.

USE OF SPONTANEOUS DRUG-RESISTANT MUTANTS TO TARGET CAPSID REGIONS IMPORTANT FOR ATTACHMENT AND UNCOATING OF PICORNAVIRUSES.

Rueckert, R.R.*, B.A. Heinz, D.A. Shepard, and W.M. Lee. Institute for Molecular Virology, University of Wisconsin, Madison, WI 53706 USA.

WIN compounds block attachment of human rhinovirus 14 (HRV14) to Hela cells apparently by deforming a specific region of the canyon floor when the drug binds in an underlying pocket. Sequence analysis of over 80 mutants resistant to high (HR) or low (LR) concentrations of drug showed that HR mutations were confined to 2 positions V188 and C199 in VP1 and the substitutions were invariably bulkier side chains. The LR mutations on the other hand occurred in a wider variety of positions, all in the drug-deformable region of VP1 on the canyon floor. All of the mutations, without exception, fell in regions involved in drug binding and virus attachment. These results suggest that drugs which block uncoating, will be similar useful for targeting capsid regions involved in the RNA uncoating process. Effects of drug resistance mutations on attachment and uncoating will be illustrated with single-cycle growth curves and plans for site-directed mutagenesis of target sites involving attachment and uncoating will be discussed.

Analysis of Coronavirus MHV surface glycoprotein functions.

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The murine coronavirus MHV-JHM has 3 envelope glycoproteins; the integral membrane protein M, the surface glycoprotein S and the haemagglutinin protein HE. It is known that the M protein plays a role in determining the intracellular site of virus maturation. The S protein mediates attachment of the virus to tissue culture cells and is involved in the fusion of viral and cellular membranes. The biological function of the HE protein is unknown.

We have taken two approaches to analysing the functions of the S and HE glycoproteins. Firstly, we have produced and mapped monoclonal antibodies which recognize linear epitopes on the S protein and neutralize virus infectivity by either preventing attachment or preventing fusion. So far, we have identified one domain responsible for attachment and two separate domains involved in fusion. Secondly, we have used the vaccinia virus system to overexpress to HE protein. It has been possible to demonstrate that this protein has both receptor binding (haemagglutination) and receptor destroying (acetylcholinesterase) activities. These results will be discussed.

ANTIGENIC STRUCTURE AND FUNCTION OF THE FLAVIVIRUS ENVELOPE PROTEIN E.

F.X. Heinz*, C. Mandl, F. Guirakhoo, H. Holzmann, W. Tuma, and C. Kunz. Institute of Virology, University of Vienna, Vienna, Austria.

The envelope protein E is of paramount significance for the biology of flaviviruses since it is essential for cell attachment and probably fusion and also induces a protective immunity after natural infection or active immunization.

We have established a structural model of the tick-borne encephalitis (TBE) virus E protein that contains information on the organization of the polypeptide chain within the protein and correlates epitopes and antigenic domains to defined sequence elements. The model is based on an epitope map that reveals the serological specificities, functional activities, and topological relationships of 19 different epitopes most of which cluster to form three major antigenic domains (A, B, and C). The structural characteristics of each epitope were determined and the localization of individual antigenic sites within the primary amino acid sequence was performed by amino-terminal sequencing of immune-reactive fragments and RNA-sequencing of antigenic variants selected in the presence of neutralizing monoclonal antibodies. Neutralization escape mutants selected by monoclonal antibodies directed to different sites were characterized with respect to their neurovirulence upon peripheral inoculation of adult mice. Mutants with a single amino acid exchange within antigenic domain B had almost completely lost their virulence. However, the mutants replicated in the mice and induced a protective immunity against peripheral challenge with virulent virus.

IMMUNE RESPONSES TO DENGUE VIRUS.

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The complex immunoregulatory responses to dengue virus are being defined using human lymphocytes, monocytes and sera from dengue infected patients. Patients with dengue have interferon gamma in their circulation at concentrations which upregulate Fc receptors on human monocytes to render them more susceptible to infection by dengue virus-antibody complexes. Dengue-immune individuals have dengue-specific memory CD4⁺ and CD8⁺ T lymphocytes. Upon stimulation during secondary dengue infection and late in primary infections, dengue specific T cells produce IFN γ . CD4⁺ and CD8⁺ T cells have specific cytotoxic activity against dengue-infected cells. MHC class I and II restricting elements present epitopes of non-structural antigens to these T cells. Interferon gamma also increases expression of MHC antigens which aids in recognition by dengue-specific cytotoxic T lymphocytes. The production and biological activity of other lymphokines, e.g. IL-1, IL-2, TNF α and IFN α have been measured in the sera of dengue infected patients. These results form the basis of a working model of the immunological regulation and pathogenesis of dengue virus infections.

CURRENT APPROACHES TO THE PROBLEM OF POLIOVIRUS ATTENUATION

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The attenuation of poliovirus is being investigated with respect to the viral mutations involved and the peculiarities of the poliovirus interaction with neural cells. The principal attenuating mutations of the Sabin vaccine strains located in the middle of the 5'-untranslated region of the viral RNA were shown to result in a diminished ability of the RNA to initiate the polypeptide synthesis in cell-free systems, suggesting the existence of a cis-acting translational control element far upstream from the initiating codon. The secondary structure of this element will be presented, as well as the results of studies on its interaction with trans-acting host protein factors. Modifications of this element as a possible way to create novel attenuated poliovirus strains will be considered. In the framework of the second approach to the attenuation problem, it was found that the reproduction of the type 1 Sabin strain, compared to its neurovirulent parent (Mahoney), in the cells of a human neuroblastoma line, was severely restricted. Both attenuated and neurovirulent polio strains were shown to be able to establish chronic infection of the neuroblastoma cells. Implications of these observations for the attenuation problem will be discussed.

THE CAPSID OF POLIOVIRUS DEPENDS ON THE MYRISTOYLATION OF VP4 FOR ASSEMBLY AND DETERMINES THE HOST RANGE OF THE VIRUS. Marc Girard, Annette Martin, Daniel Marc, Danièle Benichou, Czesław Wysocki and Sylvie van der Werf, Laboratory of Molecular Virology, Institut Pasteur, Paris, France.

Mutations were introduced by oligonucleotide cassette exchange or site-directed mutagenesis of poliovirus type 1 (PV-1) cDNA in the region coding for the viral capsid precursor P1. The properties of the resulting mutants were studied *in vivo* upon transfection of primate cells. Mutations Gly \rightarrow Arg, Ala \rightarrow Pro, and Ser \rightarrow Pro in the N-terminal sequence N(Het).Gly.Ala.Gln.Val.Ser. or P1 prevented the myristoylation of VP4 and were lethal for virus growth. Reverse mutants were found to arise spontaneously in the transfected cell cultures. All showed a restored consensus myristoylation sequence (e.g. Gly at position 1, Ala, Ser, Thr or Leu at position 2 and Ser, Thr or Ala at position 5). Lack of myristoylation did not prevent replication nor translation of the viral RNA, nor processing of P1. This suggests that myristoylation is needed at a relatively late stage of poliovirus assembly and/or for capsid stability.

Mutations of VP1 amino acids Pro95 (\rightarrow Asp), Ser97 (\rightarrow Pro) and Thr.Asn.Lys.Asp 99-102 (\rightarrow Lys.Arg.Ala.Ser) resulted in the creation of a PV-1/PV-2 chimeric virus with six amino acids from the PV-2 sequence. This virus was neurovirulent for mice. Mouse neurovirulence could be abolished by single mutations of residues Asp93 (\rightarrow Gly, Asn, Val), Asp95 (\rightarrow Gly, Asn), Lys99 (\rightarrow Asn, Glu), or Leu104 (\rightarrow Arg), or by the double mutation of Lys99 (\rightarrow Thr) and Ser102 (\rightarrow Asp) (in collaboration with T. Couderc, R. Crainic and J. Nogle). Other possible combinations are under study to determine VP1 amino acid positions critical for mouse neurovirulence. The substitution of the entire sequence of VP1 amino acids 94-102 by a foreign amino acid sequence, or its entire deletion, seemed not to alter the stability of the capsid nor the viability of the virus, while resulting in a virus with modified antigenicity. Such chimeric polioviruses could serve as the basis for new, multivalent vaccines, either live vaccines using the Sabin strain as a vector, or inactivated vaccines, using a wild-type poliovirus strain.

MOLECULAR PATHOGENESIS OF THEILER'S VIRUS INFECTION

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The Theiler's murine encephalomyelitis viruses (TMEV) are enteric pathogens of mice and members of the cardiovirus subgroup in the Picornavirus family. Two TMEV virulence groups exist: (1) Highly virulent strains which produce a lethal encephalitis in mice, and (2) Less virulent strains which cause a persistent CNS infection and chronic demyelinating disease. The demyelinating process is immune-mediated and results from a virus-specific, delayed type hypersensitivity (DTH) response rather than from autoimmune reactivity against CNS antigens or cytolytic infection of myelin-maintaining oligodendrocytes. Thus, infections by less virulent TMEV provide a relevant analog for the human disease, multiple sclerosis.

Using double-immunofluorescent staining of CNS tissues and Percoll-gradient isolated CNS mononuclear cells, TMEV was found to persist primarily in macrophages. Only 12000 macrophages are infected and only 1-5 PFU are produced/cell. The kinetics of this persistent state will be discussed.

To map elements on the genome responsible for pathogenic properties, full-length cDNA clones of two prototype TMEV strains were constructed in the bacterial plasmid pGEM3. BHK-21 cells, transfected with RNA transcripts made off the T7 RNA promoter from either the highly virulent GDVII virus clone or the less virulent BeAn 8386 virus clone produced infectious virus. The progeny viruses have the same phenotypic characteristics as the parental strains. Chimeric recombinants between the genomes of the two virulence groups have also been constructed. RNA transcripts of chimera representing mixes of the major genomic regions yield infectious virus upon transfection. The phenotypic properties of these chimeras will be discussed.

STUDIES OF ALPHAVIRUS VIRULENCE USING FULL-LENGTH CLONES OF SINDBIIS AND VENEZUELAN EQUINE ENCEPHALITIS VIRUSES
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Attenuating mutations of Sindbis and VEE have been identified after selection of parental virus populations for rapid penetration of BHK cells or resistance to neutralization by monoclonal antibodies (MCABs). The mutations were identified by sequencing, and the phenotypes of several such mutants were confirmed by inserting them into full-length clones. An ARG for SER substitution at E2 codon 114 in Sindbis strain AR339 (virulent in neonatal mice *sc* and *ic*) conferred attenuation (following *sc* inoculation), rapid penetration and increased sensitivity to neutralization by a single class of MCAB, those recognizing the E2-c antigenic site. The sequence of E2-c MCAB resistant mutants indicated that mutations at E2 62, 96, 114 or 159 affected both virulence and E2-c MCAB activity, suggesting that these residues are constituents of one pathogenesis-determining domain on the Sindbis glycoprotein spike. E2 62 and E2 114 mutations altered penetration as well as virulence. Therefore, co-selection for rapid penetration and attenuation could be explained by the presence of overlapping pathogenesis and penetration domains. In addition to mutations in E2, E1 mutations at codons 75 and 237 also affected virulence in neonatal mice. Mutants of S.A.4086 (a Sindbis strain virulent in adults *ic* as well as in neonates), which converted E2 codon 1 from SER to ASN, created a new glycosylation site and prevented PE2 cleavage. The resulting virions contained PE2 rather than E2, were rapidly penetrating and were attenuated in adults *ic* and in neonates by either the *ic* or *sc* routes. Rapidly penetrating, attenuated mutants of VEE also were isolated and sequenced. A cDNA clone of the VEE genome was constructed such that infectious VEE RNA transcripts could be derived from the clone utilizing transcription from an upstream T7 promoter. We are in the process of inserting the VEE attenuating mutations into the clone, both individually and in combination, with the hope of generating an effective, attenuated VEE vaccine candidate with a low rate of reversion to virulence.

ENTEROVIRAL HEART DISEASE

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To date, there is firm evidence from *in situ* hybridization that enterovirus infections of the human heart lead to a significant number of patients presenting with clinical signs and symptoms of myocarditis and/or dilated cardiomyopathy. In patients with dilated cardiomyopathy of recent onset, the most dramatic manifestation of myocarditis, the incidence of myocardial infection was found to be about 30%. Moreover, enterovirus RNA was not only found to be present at an early stage of the disease but also in chronic dilated cardiomyopathy, indicating persistence of the virus in the human heart. The concept of viral persistence in chronic dilated cardiomyopathy is substantiated by the finding of enterovirus persistence in follow-up biopsies of patients with ongoing cardiac disease. Another important finding was the detection of viral RNA in interstitial myocardial cells as well as in myocytes, which agrees with our previous *in vitro* findings in cultured human heart cells and persistently infected human myocardial fibroblasts. In addition, antisera raised against bacterially synthesized coxsackievirus B3 proteins are described, which revealed a broad spectrum of cross-reactivity within the enteroviruses. The use of this antisera in combination with *in situ* hybridization will allow the question of whether restricted replication is implicated in persistent forms of enterovirus-induced cardiomyopathies to be answered. Furthermore, the use of the polymerase chain reaction technique provides a powerful means to study the molecular basis of persistent enterovirus infection.

REFINEMENT OF THE SATELLITE RNA OF CUCUMBER MOSAIC VIRUS FOR USE IN THE CONTROL OF VIRUS INFECTION.
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We have shown previously that transgenic plants are resistant to infection by cucumber mosaic virus (CMV) if they express the satellite RNA of CMV. It is thought that the basis of the resistance is partly a competition between the satellite RNA and the helper virus for replicase enzymes and partly interference of the satellite RNA with the processes which lead to symptom formation.

In principle, expression of satellite RNA in transgenic plants is a highly effective strategy for control of viral infection. It is not necessary for the transgenic plants to produce new proteins. Furthermore, because the expressed satellite RNA is replicated by CMV, there is effective resistance even when the inoculum is very concentrated, or when the satellite RNA itself is expressed at a low level. However, there are also disadvantages. We have shown that the expressed satellite RNA can be acquired by viral cultures and transmitted out of the transgenic plants to non-transformed plants. This is undesirable as, in some forms, the satellite RNA can induce severe symptoms.

As a solution to these problems we have identified functional domains in the molecule so that non-essential regions can be deleted in a disabled form of the satellite RNA.

THE ROLE OF MOLECULAR EPIDEMIOLOGY IN SUPPORT OF THE GLOBAL PROGRAM TO CONTROL POLIOVIRUS INFECTIONS.
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Wild poliovirus infections paralyze nearly 250,000 children each year. The World Health Organization has initiated a global immunization program for eradicating all natural transmission of wild polioviruses by the year 2000. Important strides towards this goal have been attained in the Americas and Europe, and in parts of Asia and Africa. Laboratory support is essential to the eradication program, providing critical data on the populations at highest risk for poliovirus infection, the patterns of spread of wild polioviruses, and the identities of reservoirs sustaining transmission of wild viruses. To monitor the distribution of wild polioviruses, we have determined the genetic relationships among isolates from recent cases by partial sequencing of their RNA genomes. Since poliovirus genomes evolve rapidly, we can estimate the proximity of epidemiologic links among cases and outbreaks by determining the genetic sequence relatedness among isolates. Viruses sharing >85% of their nucleotide sequences were considered to be members of a single genotype, derived from a common ancestral infection. Many distinct genotypes (all three serotypes), having geographically defined regions of endemicity, coexist worldwide. Unambiguous links were found among cases occurring in the Middle East, Europe, North America, and South America. The genetic sequence information has been applied to the design of synthetic oligonucleotide probes of predetermined specificities (vaccine- or wild genotype-specific), permitting surveillance for wild genotypes by simple, routine diagnostic tests.

RATIONAL DESIGN OF ANTIPICORNAVIRAL AGENTS.

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A novel class of antipicornaviral agents discovered by the Sterling Research Group have been shown to inhibit the replication of a broad spectrum of human rhinoviruses and enteroviruses in vitro and prevent enterovirus-induced paralysis when administered orally to infected mice. Initial mechanism of action studies demonstrated that these compounds blocked uncoating of rhinovirus type-2 (HRV-2) and poliovirus type-2 via a direct interaction with the virion. In the absence of data on the molecular details of the binding site for these molecules, conventional structure-activity relationship analysis was successfully applied to the design of compounds with improved potency and spectrum of activity. Following the elucidation of the binding site for these compounds at atomic resolution within rhinovirus type-14 (HRV-14) by the Purdue group headed by Michael Rossmann, it was for the first time possible to begin true rational design of compounds with enhanced potency. The x-ray structure revealed that the drug binding pocket was predominantly hydrophobic in nature, and design efforts have been directed towards compounds which would increase hydrophobic interactions through filling empty space in the pocket. The conformational changes induced in VP1 following compound binding were found to extend to residues within the floor of the "canyon", or proposed cellular receptor (ICAM-1) binding site. Subsequent analysis showed that HRV-14 and other major receptor group rhinoviruses examined are all inhibited in their ability to attach to the cellular receptor, thus providing support for the canyon as the receptor binding site. Minor receptor group virus examined thus far, including HRV-2, are not blocked in attachment, and are all presumably inhibited at the uncoating step.

REDESIGNING POLIOVIRUSES FOR VACCINE PURPOSES.

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With the objective of developing improved polio vaccines and oral vaccines against other diseases, we have used site-directed mutagenesis to modify the genomes of the Sabin vaccine strains of poliovirus types 1 and 3. Studies on type 3 have concentrated on modifications of the 5' non-coding region based on a secondary structural model derived by computer modelling and biochemical analysis (Skinner et al. 1989). Our results suggest that both the primary and secondary structure of the 5' NCR can influence the neurovirulence of the virus.

Our studies on poliovirus type 1 have concentrated on modifications to antigenic domains. We have previously shown (Burke et al. 1988) that type 3 antigenic domains can be correctly expressed on the type 1 particle. We have extended these studies to antigenic sites 2 and 3 and have introduced antigenic domains from heterologous viruses such as HPV-16 and HIV (Evans et al. 1988). The antigenic and immunogenic properties of these virus chimaeras will be discussed.

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SEQUENCE ANALYSIS OF THE GENOME RNA OF LACTATE DEHYDROGENASE-ELEVATING VIRUS (LDV).
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LDV was initially classified as a togavirus on the basis of its morphology and genome type, namely a single-stranded RNA of positive polarity. Further characterization of the genomic RNA revealed that it contained a 3' poly A tract as do the genomes of other togaviruses. However, in contrast to togaviruses, no subgenomic viral mRNA has been observed in LDV-infected cells. Randomly primed cDNA obtained from the genome of the neurotropic LDV isolate, LDV-C, cross-hybridized with the genomes of other LDV isolates, but not with the genomes of an alphavirus, rubella virus, a flavivirus, or cellular rRNA. Sequence information obtained from cDNA synthesized from LDV RNA indicated that the sequence of the 3' terminal genomic region is highly conserved among different isolates of this virus. However, the LDV 3' terminus does not contain highly conserved sequences or RNA secondary structures characteristic of the 3' termini of either the togavirus or flavivirus genomic RNAs. Additional information obtained upon completion of the LDV-C genome sequence analysis and the mapping of the virion structural proteins will determine whether LDV should be reclassified as a new family or remain within the Togaviridae.

INSERTION OF UBIQUITIN CODING SEQUENCE IDENTIFIED IN THE RNA GENOME OF A TOGAVIRUS
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Pestiviruses, currently classified as togaviruses, are positive stranded RNA viruses which represent causative agents of severe animal epidemics. Comparison of the genomic sequences of different members of the genus pestivirus, in particular two bovine viral diarrhoea virus (BVDV) strains and a hog cholera virus (HCV) strain, led to the identification of strain specific insertions in the BVDV genomes. Such insertions are not present in the HCV sequence. The amino acid sequence encoded by the insertion of one BVDV strain (Osloss) and the conserved animal ubiquitin sequence are almost identical. For the insertion in the genome of a second BVDV strain (NADL) homology to a host cellular mRNA was demonstrated by hybridization. The nucleotide sequence of a cDNA clone derived from cellular mRNA exhibits 99% homology to this BVDV insertion. These findings which can be explained by an RNA recombination process between viral and host cellular sequences add a new aspect to the evolution of RNA viruses. In addition they lead to a novel model for pathogenesis of a persistent pestivirus infection.

ASSOCIATION OF ALPHAVIRUS REPLICATION WITH THE CYTOSKELETAL FRAMEWORK AND TRANSCRIPTION *IN VITRO* IN THE ABSENCE OF MEMBRANES.

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Alphavirus (Sindbis or Semliki Forest virus) RNA synthesis occurs in complexes that are composed of the viral nonstructural proteins (nsPs), viral RNA templates and possibly associated host factors. These replication complexes are associated with the smooth membrane fraction of infected cells. An obstacle to the purification of alphavirus replication complexes has been the inability to remove the membranes and retain the RNA polymerase activity *in vitro*. Because treatment of the membrane fraction with detergents releases endogenous RNases, it proved difficult to assess the polymerase activity of detergent treated replication complexes. We developed a method to measure the polymerase activity of detergent treated membranes that did not depend on measuring the incorporation of radioactive precursors into the single-stranded RNA products of the viral replicase. Using this method we have obtained from the smooth membrane fraction (1.16g/cc) of infected cells membrane-free replication complexes that synthesize *in vitro* viral RNA. Removal of the lipids with Triton X-100 and deoxycholate caused the density of the replication complexes to shift from 1.16 g/cc to 1.25g/cc. The membrane-free replication complexes retained at least 80% of the polymerase activity associated with the membrane associated replication complexes. Although membrane-free, the detergent treated complexes co-purified with the detergent-insoluble cytoskeletal framework. Immunoprecipitation with monospecific antibodies (gift of R. Hardy and J. Strauss) identified nsP1, nsP2, nsP3 and nsP4 in the lipid-free fraction. Therefore, alphavirus replication complexes do not require lipids for polymerase activity *in vitro* and appear to interact with the cytoskeletal framework of infected cells.

DEFINED MUTATIONS IN THE POLIOVIRUS CAPSID PROTEINS CAUSE SPECIFIC DEFECTS IN RNA ENCAPSIDATION, RNA UNCOATING AND VP0 CLEAVAGE.

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We have constructed, isolated and characterized two poliovirus mutants, VP1-101 and VP1-102, whose phenotypic defects are caused by two different small deletions in the amino terminus of VP1. The lesions were introduced by a random deletion mutagenesis procedure. The amino terminus of VP1 is unresolved in the three-dimensional structure of the poliovirion, but is known to be buried within the virion and is likely to be interacting with the viral RNA. Both VP1-101 and VP1-102 show a diminished ability to enter CV1, but not HeLa, cells. Neither the rate of binding to cells nor of subsequent "alteration" of the mutant poliovirions is affected; the defect in cell entry can be traced to a lower rate of uncoating of the viral RNA in the mutant viruses on CV1 cells. Although the phenotype of VP1-101 can be amply explained by this single defect, VP1-102 displays an additional biochemical deficiency. In VP1-102-infected CV1 cells, 75S empty capsids and 14S intermediates accumulate, but 135S "provirions" or 150S infectious virions are not observed. Thus, only those subviral structures that do not include viral RNA are seen. We suggest that the mutation in VP1 in VP1-102 affects RNA encapsidation as well as uncoating, and that these two related processes both involve the amino terminus of VP1. The temperature-sensitive defect of another poliovirus mutant, VP2-103, is caused by a single nucleotide substitution in the VP2 coding region. At nonpermissive temperatures, 100-fold fewer infectious virions are observed. Instead, structures sedimenting at 135S that are in every respect identical to the elusive "provirion" are produced. Furthermore, the proteolytic cleavage of VP0, a reaction that has been postulated to be autoprolytic and RNA-dependent, is not observed at high temperatures in this mutant. We suggest that the primary defect of VP2-103 is in VP0 cleavage.

ANTIVIRAL COMPOUNDS DISTINGUISH BETWEEN TWO SUBGROUPS OF RHINOVIRUSES WITH DIFFERENT BIOLOGICAL PROPERTIES
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A variety of chemically different compounds inhibit the replication of several serotypes of rhinoviruses (common cold viruses)⁽¹⁾. We noticed that one of these compounds, WIN 51711, had an antiviral spectrum clearly distinctive from a consensus spectrum, identified for other capsid-binding compounds. On the other hand, mutants resistant to R 61 837, an antiviral pyridazinamine, were shown to be cross-resistant to all other capsid binding compounds, including WIN 51711⁽¹⁾. This indicated that compounds although sharing the same binding site could have different spectra of antiviral activity.

A systematic evaluation of 15 known rhinovirus-capsid binding compounds against all serotyped rhinoviruses was therefore initiated. Multivariate analysis⁽²⁾ of the results revealed the existence of a major and a minor drug subgroup of rhinoviruses, exhibiting differential susceptibility to antiviral compounds and suggesting the existence of a dimorphic binding site.

The observed relationships between serotypes (regarding their antiviral susceptibility) turned out to be highly correlated with sequence data⁽³⁾ of amino acids, not only of the putative binding site (17 amino acids) but also of VP1 (280 amino acids) and even of other genome regions. Rhinoviruses belonging to the minor receptor subgroup all cluster in the major drug subgroup. Furthermore, we found convincing evidence to allow for the conclusion that serotypes belonging to the major drug subgroup produce more than twice as many clinical infections than viruses belonging to the minor drug subgroup.

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CHARACTERIZATION OF SEQUENCE VARIATION AMONG ISOLATES OF RNA VIRUSES: THE DETECTION OF MISMATCHED CYTOSINE AND THYMINE IN RNA-DNA HETERODUPLEXES BY CHEMICAL CLEAVAGE

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An end-labelled cDNA probe of known sequence was prepared from the PUO-218 isolate of dengue virus type 2 (DEN-2) and used to form RNA-DNA heteroduplexes with viral RNA of two other DEN-2 isolates, namely New Guinea C and D80-100. The nucleic acids were specifically cleaved at mismatched cytosine and thymine bases following treatment with hydroxylamine and osmium tetroxide respectively. The points of cleavage in the end-labelled DNA probe were located by electrophoresis of the heteroduplex samples through denaturing gels in parallel with standard Maxam and Gilbert reactions of the same probe. The chemical cleavage method used in this study is of general application to the study of variation in the nucleotide sequences of RNA viruses. Provided appropriate cDNA probes are available, significant regions of a genome can be specifically targeted for analysis. In some instances, such as epidemiological surveys, a "fingerprint" of difference for a region using a probe of one sense only may be sufficient. Alternatively, more detailed information can be obtained using DNA probes of both senses against positive and negative strand RNA.

STUDIES ON THE MECHANISM OF READTHROUGH SUPPRESSION IN MOLONEY MuLV.

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In Moloney (Mo-)MuLV, an in-frame UAG termination codon separates the gag and pol genes. ~95% of the translation products end at the UAG, but in the remaining 5% the UAG is read as Gln¹. We have studied the mechanism of readthrough in Mo-MuLV, using both *in vitro* translation and experiments *in vivo*. We find (1) contrary to a previous report², the suppression is mediated by a normal, rather than virus-induced or altered glutamine tRNA. (2) Mutants containing UAA or UGA in place of UAG are also suppressed, both *in vitro* and *in vivo*, and are fully infectious. Thus, mammalian cells contain tRNAs capable of inserting amino acids in response to UAA and UGA as well as UAG, and the signal required for UAG suppression is also effective for the other two stop codons. While suppression of UGA is well known, mammalian suppression of UAA has only been documented in one other case (Sindbis virus³). We are now attempting to identify signals involved in suppression. Computer analysis suggests the existence of several stem-loop structures near the UAG in Mo-MuLV RNA, but our experiments to date have not shown any of them to be sufficient for suppression. We are also attempting to identify the amino acids inserted in response to UAA and UGA.

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FOOT-AND-MOUTH DISEASE VIRUS PROTEASE 3C INDUCES SPECIFIC PROTEOLYTIC CLEAVAGE OF

HOST CELL HISTONE H3

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In foot-and-mouth disease virus (FMDV) infected cells disappearance of the nuclear protein histone H3 and the simultaneous appearance of a new chromatin associated protein termed P1 can be observed. We have sequenced the amino terminus of P1 and clearly showed that it derives from histone H3 by proteolytic cleavage. The 15 N-terminal amino acid residues are specifically cleaved off early during infection. In addition using an *in vitro* transcription/translation assay with different FMDV clones we showed that the histone H3 - P1 transition is catalysed by the FMDV 3C protease, which until now has only been found to be responsible for the processing of the viral polypeptide. The 3C protease is the only FMDV protein required to induce this histone H3 - P1 transition. As the deleted part of the histone H3 corresponds to the domain presumed to be involved in the regulation of transcriptional active chromatin in eucaryotes, it is postulated that this specific cleavage of H3 is a mechanism which FMDV utilises to switch off host cell RNA synthesis, as is reported for picornaviruses.

ANALYSIS OF A SECOND PROTEASE IN HUMAN RHINOVIRUSES

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Evidence is presented that the protein 2A of human rhinovirus serotype 2 (HRV2) is a protease. On expression of the VP1-2A region of HRV2 in bacteria, protein 2A was capable of acting on its own N-terminus. Deletion experiments showed that removal of 10 amino acids from the carboxy terminus inactivated the enzyme. Site-directed mutagenesis identified an essential arginine close to the C-terminus and showed that the enzyme was sensitive to changes in the putative active site. This analysis supports the hypothesis that 2A belongs to the group of sulphhydryl proteases, although sequence comparisons indicate that the putative active site of HRV2 2A is closely related to that of the serine proteases.

CHIMERIC HEPATITIS B VIRUS CORE PARTICLES CONTAINING DENGUE-2 ENVELOPE PROTEIN EPITOPES INDUCE DENGUE VIRUS NEUTRALIZING ANTIBODY

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Defining major immunodominant regions of the dengue (DEN) virus envelope glycoprotein has facilitated development of flavivirus subunit vaccines. Immune responses were elicited with free synthetic peptides derived from the amino acid sequence of the DEN virus envelope glycoprotein administered in Freund's complete adjuvant. However, because the immunogenicity of the free peptides was much lower than that of the whole virus, we constructed chimeric hepatitis B virus core particles (HBcAg) into which DEN virus envelope protein epitopes were engineered into the amino-terminal end of the core protein. Six DEN-2 amino acid sequences, representing antigenically important epitopes in the R1, R2, and R3 domains of the envelope protein were engineered into HBcAg. Outbred and inbred mice immunized with purified chimeric HBcAg produced antibodies that reacted by ELISA with DEN-2 virus and synthetic peptide equivalents of the inserts. Antibodies produced to the chimeric particle which contained the DEN envelope sequence from amino acid 35 through 55, neutralized virus infectivity *in vitro*. DEN virus envelope protein epitopes presented on the surface of the HBcAg are extremely immunogenic and potentially useful as subunit vaccines for prevention of flavivirus disease.

PROPER PROCESSING OF DENGUE VIRUS NONSTRUCTURAL GLYCOPROTEIN NS1 REQUIRES THE N-TERMINAL HYDROPHOBIC SIGNAL SEQUENCE AND THE DOWNSTREAM NONSTRUCTURAL PROTEIN NS2a

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Expression of dengue viral gene products involves specific proteolytic cleavages of a precursor polyprotein. To study the flanking sequences required for expression of the dengue virus nonstructural glycoprotein NS1, we constructed a series of recombinant vaccinia viruses that contain the coding sequence for NS1 in combination with various lengths of upstream and downstream sequences. The NS1 products expressed by these viruses in infected CV-1 cells were immune precipitated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The data show that the 24-residue hydrophobic sequence preceding NS1 was necessary and sufficient for the production of glycosylated NS1, and that this sequence was cleaved from NS1 in the absence of most dengue viral proteins. This finding is consistent with previous proposals that this hydrophobic sequence serves as an N-terminal signal sequence that is cleaved by signal peptidase. The cleavage between the C-terminus of NS1 and the downstream protein NS2a occurred when the complete NS2a was present. Recombinant viruses containing NS1 plus 15 or 49% of NS2a produced proteins larger than authentic NS1, indicating that the cleavage between NS1 and NS2a had not occurred. Failure of cleavage was not corrected by co-infection with a recombinant virus capable of cleavage. These results suggest that NS2a may be a *cis*-acting protease that cleaves itself from NS1, or NS2a may provide sequences for recognition by a specific cellular protease that cleaves at the NS1-NS2a junction.

DEFINITION AND TRANSLATION OF THE FLAVIVIRUS POLYPROTEIN

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The single long open reading frame of the flavivirus genomic RNA codes for a polyprotein of about 3400 amino acids. By nucleotide sequencing of cloned cDNA of Kunjin (KUN) virus and N-terminal amino acid sequencing of infected cell products, we have positively identified all the encoded polypeptides and defined the gene order 5'-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3', including the boundaries of all 10 genes. The C-prM-E cleavages occur via a signal peptidase. The remaining cleavages occur at two consensus sites common to all flaviviruses: Val-X-Ala^x preceding NS1, NS2a and NS4b, and a site containing two basic amino acids (defined originally by C. Rice and colleagues) preceding NS2b, NS3, NS4a and NS5. The proteases remain unidentified.

Recombinant vaccinia viruses (recVV) containing KUN virus genes expressed the structural proteins prM and E and the nonstructural proteins NS1, NS3 and NS5 (shown by immunofluorescence). The single gene products NS3 and NS5 were each radiolabelled in cells infected with the corresponding recVVs. Multigene expression from cDNA of NS3-NS5 was detected only as a full length labelled product in polyacrylamide gels. Radiolabelled E was cleaved correctly when expressed from the cDNA sequence C-NS2b, either in recVV infected cells or during *in vitro* translation from SP6 polymerase transcripts with membranes added.

LOCALIZATION OF NEUTRALIZATION EPITOPES IN
THE GLYCOPROTEINS OF SINDBIS VIRUS BY
ANALYSIS OF ANTIBODY ESCAPE VARIANTS

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We have been interested in localizing the antigenic epitopes on the glycoproteins of an alphavirus, Sindbis virus, which are involved in virus neutralization. A panel of neutralizing monoclonal antibodies (MAbs) were used to select Sindbis variants which no longer reacted with the antibodies. To localize the antigenic epitopes present on glycoprotein E2, eight resistant variants, as well as 4 revertants which had regained reactivity, were sequenced throughout the E2 region. In addition 4 isolates, selected for resistance to an anti-E1 neutralizing MAb were sequenced throughout the E2 and E1 regions. All of the salient changes in E2 occur within a relatively small region between amino acids 181 and 216, near the conserved glycosylation site. Variants independently isolated for resistance to the same MAb were usually altered in the same amino acid, although MAb 50 selected for changes at two different residues. Resistance to the single E1-specific MAb resulted from changes at Gly-132 of E1 to either Arg or Glu. Reversion occurred at the sites of the original mutations, but did not always restore the parental amino acid.

P 1

A COMMON LEADER SEQUENCE IS SPLICED TO ALL SUBGENOMIC RNAs OF EQUINE ARTERITIS VIRUS.

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During the replication of equine arteritis virus (EAV), 6 intracellular subgenomic RNAs are synthesised by processing of genome length precursor RNA. Sequence and northern blot analysis indicated that the viral RNAs form a 3'-coterminal nested set, similar in organisation to coronaviral RNAs. Oligonucleotide hybridisation analysis and primer extension experiments showed that the smallest RNAs (RNA 6 and 5) are not colinear with the 3'-end of the genome. Sequence analysis of cDNA clones derived from total intracellular RNA revealed a leader sequence of 208nt at the 5'-ends of RNAs 5 and 6. A leader specific probe hybridised in colony blots to a clone mapped to the 5'-end of the genome and in northern blots to each subgenomic RNA. Sequence analysis of the 5'-end of the genome positively identified it as the origin of the leader sequence; the sequence at the 3'-end of the leader (AUCUCUA) and of an area 50nt downstream (UUUGGAGGG) are almost identical to the sequence of the Tetrahymena rRNA 5'-splice site and internal guiding sequence respectively. The first 5 nucleotides (TGACC) of the body of EAV RNAs 6 and 5 are identical. The same sequence was found at the 3'-end of the leader sequence (at the 5'-end of the genome and just upstream of ORF1 and 4 on the genome). These data suggest that splicing at multiple internal sites produces EAV subgenomic mRNAs with a common 5'-leader sequence.

P 2

IN VITRO SYNTHESIS OF INFECTIOUS VENEZUELAN EQUINE ENCEPHALITIS VIRUS RNA FROM A cDNA CLONE: ANALYSIS OF A VIABLE DELETION MUTANT

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A cDNA clone of Venezuelan equine encephalitis virus (VEE) was constructed and used as the template for synthesis *in vitro* of infectious RNA genomes. A T7 promoter directed RNA synthesis to begin at a G residue followed by the exact 5'-terminus of the VEE genome. Transcripts were specifically terminated just beyond the poly (A) tract by using templates digested at a unique Not I site in this region. RNA transcripts of the cDNA clone, but not the clone itself, were able to initiate a productive infection of DEAE dextran-treated chicken embryo fibroblasts (CEF). VEE antigens were demonstrated in RNA-transfected cells, and supernatants from transfected cultures contained infectious virus particles. The cDNA clone lacked 102 nucleotides of the vEE genome sequence. The deletion, which also was present in the genomes of progeny virions derived from the clone, did not appear to affect growth in cultured CEF, baby hamster kidney or Vero cells, or the virulence of progeny virions in mice. The site of the deletion was mapped to the 3'-end of the nsP3 gene by comparison to other alphavirus sequences. In this region, the VEE genome sequence includes two tandem 102-nucleotide repeats which can be arranged in a stable stem and loop structure. The sequence remaining in the deleted clone retains one copy of the duplicated sequence and, in addition, faithfully preserves a portion of the predicted stem.

P 3

ANALYSIS OF THE ROLE OF ICR-LIKE SEQUENCES IN SYNTHESIS OF (+) STRAND GENOMIC RNA

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The genomic RNAs of BMV (brome mosaic virus) possess sequences at their 5' termini which resemble the internal promoters of eukaryotic tRNAs. These sequences are found as two blocks corresponding to the ICRs (internal control regions) 1 and 2 (also called A and B boxes) of tRNA genes. In comparison with the tRNA consensus sequence, the 5' ICR2 or B box like sequence is well conserved on BMV RNAs 1 and 2, whereas the 5' ICR-like sequences are not as conserved on genomic RNA3. However genomic RNA3 also possesses two internal regions of ICR-like sequences with the more conserved ICR2-like sequence found in the intercistronic region. In order to study the role of the ICR-like sequences of RNAs 1 and 2 in (+) strand RNA synthesis, a deletion mutant (or ΔRNA2) of RNA2 has been used which replicates analogously to RNA3 in requiring the presence of wild type RNAs 1 and 2. Oligonucleotide site directed mutagenesis has been utilized to make specific deletions and substitutions in cloned cDNAs from which infectious RNAs can be transcribed *in vitro*. Such deletions indicate that the 5' ICR2-like sequences of RNA2 make a significant contribution to (+) strand RNA synthesis. Similar deletions have also been made on genomic RNA3. In contrast with that of RNA2, the 5' ICR2-like sequences of RNA3 do not appear to contribute significantly to (+) strand RNA synthesis. Instead the more conserved intercistronic ICR2-like sequence appears to make a greater contribution to (+) strand RNA synthesis.

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INFLUENCE OF 3' TERMINAL MUTATIONS ON *IN VIVO* REPLICATION OF BROME MOSAIC VIRUS RNA2.

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Each of the four virion RNAs of brome mosaic virus (BMV) possess a tRNA-like structure at the 3' end which is responsible for the specific tyrosylation of the 3'CCA^{OH} terminus. Additionally this structure interacts with host nucleotidyl transferase and functions as the promoter for initiation of (-) strand synthesis. In order to elucidate the role of tRNA mimicry in viral replication, we have introduced several modifications encompassing the entire tRNA-like region and identified a valuable pool of mutants that can be subjected to *in vivo* analysis. Three mutant sequences (5'PsK, 5'+3'PsK and ΔK.nob), previously been characterized to be defective in one or more tRNA associated activities, have now been analyzed for their effect on *in vivo* replication of BMV RNA2 that is integral to the life cycle of the virus. Capped full length mutant RNA2 transcripts synthesized *in vitro* were mixed with wild type transcripts of RNA1 and RNA3. When inoculated to barley protoplasts, all RNA2 mutants replicated very poorly (<5%) but did not interfere with replication and accumulation of other RNA components. Additional experiments with protoplasts confirmed that the factor encoded by RNA2 acts *trans* and is only required in catalytic amounts. Inoculation of mutant transcripts to *Chenopodium hybridum* induced necrotic local lesions characteristic of wild type BMV infection. To verify the progeny RNA2 in each mutant infection, single lesions were isolated and propagated in barley plants. Sequence analysis of progeny RNA2 indicated that input mutations were restored to wild type sequences derived from 3' homologous region of RNA3, presumably by recombination events.

INFECTIOUS RNA FROM A FULL-LENGTH cDNA CLONE OF CUCUMBER NECROSIS VIRUS. D.M. Rochon. Agriculture Canada Research Station, Vancouver, B.C. CANADA V6T 1X2.

Cucumber necrosis virus (CNV), a tombusvirus, is a simple spherical virus ca. 30 nm in diameter which contains a positive polarity RNA genome 4.7 kb in length. Virion RNA is probably capped at the 5' terminus and lacks a 3' poly(A) tail. The complete genomic sequence of CNV has been determined. A full-length DNA copy of CNV was constructed and placed downstream from the T7 promoter in the phagemid Bluescribe (Stratagene). Transcription using T7 RNA polymerase resulted in transcripts which were infectious when inoculated to several CNV hosts. Infectivity estimates using the local lesion host *Chenopodium amaranticolor* showed that capped synthetic CNV transcripts were ca. 0.5% as infectious as natural virion RNA. Virus derived from plants inoculated with the synthetic transcripts, however, was as infectious as the authentic virus. The 5' and 3' termini of the synthetic transcripts contain 4 and 1 additional non-viral nucleotides, respectively. Experiments are in progress to determine if infectivity of the synthetic transcripts can be improved by removal of 5' non-viral nucleotides.

NUCLEOTIDE SEQUENCE OF THE 3' TERMINAL REGIONS OF TOMATO RINGSPOT VIRUS RNA-1 AND RNA-2. M.E. Rott, J.H. Tremaine and D.M. Rochon*. Agriculture Canada Research Station, Vancouver, B.C. CANADA V6T 1X2.

Tomato ringspot (TomRSV) is a member of the nepovirus group which forms part of the picornavirus-like superfamily. Both genomic components of the bipartite TomRSV were cloned and partially sequenced. The nucleotide sequences were determined of ca. 2.5 kb and 5.5 kb at the 3' ends of RNA-1 and RNA-2, respectively. Comparisons of the 3' proximal nucleotide sequences of RNA-1 and RNA-2 demonstrated near identity over an extended region (1534 of 1535 nt). The shared regions were devoid of long open reading frames and thus are unlikely to have coding functions. The sequences in RNA-1 and RNA-2 upstream from the shared regions each contained a single long open reading frame. The deduced amino acid sequence in RNA-1 showed strong similarity with the putative replicases of picorna-like viruses. A portion of the upstream sequence in RNA-2 showed amino acid sequence similarity with the coat protein of the nepovirus tomato black ring. The region upstream from the putative TomRSV coat protein contained two sets of direct repeats which preserved the single long open reading frame in RNA-2. Efforts are being made to complete the sequence and structure of the TomRSV genome.

GENOME STRUCTURE AND EXPRESSION OF BERNE VIRUS, THE PROTOTYPE TOROVIRUS.

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Berne virus (BEV) is the prototype of a newly proposed family of positive-stranded animal RNA viruses, the Toroviridae. The BEV genome, a polyadenylated RNA molecule of 25-30 kb, is surrounded by a tubular nucleocapsid and a peplomer-bearing envelope. In BEV-infected cells a genome-sized RNA and 4 subgenomic viral RNAs are synthesized. Northern blot analysis showed that the BEV mRNAs form a 3'-coterminal nested set. In vitro translation of purified BEV mRNAs and sequence analysis of mRNA-derived cDNA clones revealed the gene order 5'-polymerase - peplomer protein - small envelope protein - nucleocapsid protein - 3' along the genome.

UV transcription mapping data demonstrated that the BEV RNAs are transcribed independently. By primer extension and oligonucleotide hybridizations the smallest BEV RNA (5) was found to be contiguous on the consensus sequence. The nucleotide sequence surrounding the potential transcription initiation site shows a high degree of similarity to sequences upstream of other ORFs. This suggests the presence of four subgenomic RNA promoters on the template.

Except for the polymerase gene product (see abstract P.J. Bredenbeek et al.), no amino acid similarities between toro- and coronavirus gene products were observed. Hence, our data on the torovirus genome structure justify the proposal of a new virus family (Horzinek (1987); Intervirology 27: 17-24).

SEQUENCE OF THE NONSTRUCTURAL PROTEINS OF TICK-BORNE ENCEPHALITIS VIRUS (WESTERN SUBTYPE) AND COMPARATIVE ANALYSIS WITH OTHER FLAVIVIRUSES

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Tick-borne encephalitis (TBE) virus (Western subtype vaccine strain Neudoerfl) was cloned and the nucleotide sequence coding for all nonstructural proteins (approximately 8 kb) was determined. The amino-termini of the individual proteins were assigned by comparison with other flavivirus sequences. Amino acid homology calculations between TBE virus and mosquito-borne flaviviruses were performed for all nonstructural proteins. An evolutionary tree based on protein NS1 is presented that reveals the molecular basis of relationships among flaviviruses. Tick-borne and mosquito-borne flaviviruses share a common hydrophilicity profile and also other features of their primary sequences, such as the presumably functional Gly-Asp-Asp sequence element within protein NS5. Other characteristics, such as the potential N-glycosylation sites of protein NS1 and a potential proteolytic cleavage site within protein NS4B, are conserved within the mosquito-borne group, but differ in the TBE virus sequence.

CIS-ACTING ELEMENTS INVOLVED IN REPLICATION OF ALFALFA MOSAIC VIRUS RNAs.

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The genome of alfalfa mosaic virus consists of 3 RNA molecules. Coat protein is translated from a subgenomic mRNA (RNA4) derived from RNA3. An RNA-dependent RNA-polymerase (RdRp), isolated from infected bean plants, specifically initiated (-)RNA synthesis on a (+)RNA4 template and RNA4 synthesis on a (-)RNA3 template in an *in vitro* assay. Using T7-transcripts of mutated cDNA3 as template, the RdRp recognition sites involved in the initiation of (-)RNA and (+)RNA synthesis were identified. Surprisingly, the extreme 3'-ends of (+)RNA templates are not involved in this recognition.

Transcripts of cDNAs 2 and 3 were infectious and permitted a study of the RNA replication *in vivo*. The results differ substantially from those obtained with brome mosaic virus.

VARIABILITY AND EVOLUTION OF FIELD ISOLATES OF PLANT RNA VIRUSES.

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Quantitative studies on the genetic variation of plant RNA viruses are very scarce, in spite of their theoretical and applied importance. We report here on the genetic variability and evolution of field isolates of the plant RNA virus U5-TMV naturally infecting the wild plant *Nicotiana glauca* Grah. The populations studied were composed of a high number of haplotypes, that seemed to be selectively neutral according to Ewen-Watterson's test. Two main features are found regarding U5-TMV evolution: 1st) there is no correlation between genetic proximity of isolates and geographic proximity of the sites from which they were obtained; 2nd) the estimated divergence among haplotypes is low, and values are maintained no matter the scale of the distance between the sites from which the isolates come. No comparable studies have been done with a plant RNA virus, and these two features seem to be unique for this system as compared with other RNA viruses.

The distribution of the observed genetic variation on the different regions of the genome has been studied in an attempt to understand the functional significance of this evolutionary model. Conserved and variable domains were found not correlating with viral genes.

CLONING AND CHARACTERIZATION OF THE HOG CHOLERA VIRUS IN AN EXPRESSION VECTOR.

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Hog Cholera Virus was obtained and concentrated 100 times by ultrafiltration (Millipore) of the spent medium of HCV infected PK-15 cells grown in 6-wtcdex 3' microcarrier giving a final titer of 5×10^6 TCID₅₀/ml. The RNA was extracted using the hot phenol/guanidinium method and was used as template for the synthesis of complementary DNA by the method of Gubler & Hoffmann. The cDNA was cloned into the vector arms of λ gt11 using EcoRI linkers and recombinant phages grown in *E. coli* Y1090 were screened with porcine hyperimmune serum against HCV (B. Liess, Hannover, FRG) using the biotinylated protein A - streptavidin horseradish peroxidase system. Four out of 50,000 plaques screened reacted positively to the serum. Each of the recombinant phages contained an insert of less than 300 bp which hybridized specifically with a high molecular weight RNA band in a Northern blot with RNA from HCV-infected PK-15 cells. Crude protein extracts were prepared from *E. coli* Y1089 lysogens and the fusion proteins were purified by anti β -galactosidase immunoaffinity adsorption. The purified fusion protein reacted positively to HCV antisera but not negatively to Bovine Viral Diarrhea Virus antisera.

TORO- AND CORONAVIRUSES: A NEW SUPERFAMILY OF POSITIVE STRANDED RNA VIRUSES.

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Toro- and coronaviruses both are enveloped positive-stranded RNA viruses containing a large genomic RNA (25-30 kb). Similarities between both virusfamilies in genome organization such as the order and the types of genes along the genome and the presence of multiple subgenomic RNAs have recently been established. Besides these common features there are significant differences in the replication strategy of both virus families (see abstract E. J. Snijder et al.).

The polymerase (pol) gene of IBV is the only coronaviral pol gene whose sequence has been published (Bournsell et al., J. Gen. Virol. 58, 57-77); no torovirus sequences have yet been published. Recently we have sequenced a substantial part of the pol genes of the coronavirus MHV-A59 and the torovirus BEV. The MHV sequence revealed at least two ORFs. In contrast to the absence of any noticeable conservation in the amino acid sequence of the first ORF of the pol gene, the predicted amino acid sequence of the second ORF was well conserved between MHV and IBV. Comparison of the predicted amino acid sequence from the 3' part of the pol gene of BEV with the product encoded by ORF2 of the coronavirus pol gene revealed striking similarities. These data seem to justify the classification of toro- and coronaviruses into a new superfamily.

MOLECULAR CLONING AND EXPRESSION OF A RNA-DEPENDENT RNA POLYMERASE OF PLUM POX VIRUS IN ESCHERICHIA COLI

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The complete nucleotide sequence of Plum Pox potyvirus (PPV) shows one single open reading frame, coding for a protein of Mr 354 kD¹. The amino acid sequence shows similarities to other viruses with different degree of homology, depending on the respective genes.

The predicted amino acid sequence of the N1b-like region, located adjacent to the coat protein (at the C-terminus²) of the polyprotein shows homologies of 61.3% to the respective protein of Tobacco Vein Mottling Virus and 55% to that of Tobacco Etch Virus. A typical polymerase sequence motif³, RYFVNGDDLVLAV, can be identified in this protein. To identify the function of this N1b-like protein we have cloned the gene into the E. coli expression vectors and purified the recombinant protein

- ¹ Maiss et al. (1989) J. Gen. Virol. 70 513
- ² Mattanovich et al. (1988) Virus Genes 2 119
- ³ Argos P. (1988) Nucleic Acids Res. 16 9909

MOLECULAR CLONING OF DEFECTIVE-LIKE RNA OF TWO HORDEIVIRUSES

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Hordeiviruses, type member is barley stripe mosaic virus (BSMV), have three genomic RNAs which are encapsidated separately in rod-shaped virions. One strain of BSMV contains a defective RNA (RNA 4), which is originated from RNA 3 (McFarland et al., 1983).

We found a similar, extra RNA (RNA 4) in purified virions of two other hordeivirus isolates, i.e. the Type strain of pea semilant virus (PSLV) and M1-Da2 strain of lychyns ring-spot virus (M1-Da2). Genomic RNAs as well as RNA 4 of both viruses were isolated and then copied into cDNA and cloned into pUC 18. Clones corresponding to each RNA were identified.

Northern blot analysis of encapsidated RNAs of both viruses showed that their RNA 4 is not originated from RNA 3, as in the case of BSMV. Clones of RNA4 of M1-Da2 hybridized only to RNA 2 and 4, but not to 1&3. RNA 4 of PSLV was found to show great sequence homology with RNA 1 or 2 (RNA 1&2 are poorly resolved by denaturing agarose gel electrophoresis), but not to RNA 3.

THE 5'-TERMINUS OF MURRAY VALLEY ENCEPHALITIS VIRUS RNA IS CONSERVED AND FORMS A STABLE SECONDARY STRUCTURE.

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The 5' non-coding region of the genomes of 11 isolates of Murray Valley encephalitis virus (MVE) from Australia and Papua New Guinea were examined by primer extension sequencing. It was found that the 5' non-coding region of all isolates was highly conserved. The two isolates from Papua New Guinea contained an extra uridine residue, nominally positioned after nucleotide 54, which was absent from all but one of the Australian isolates tested. This isolate (OR 156) contained a further uridine residue at the same site. It should be noted that OR 156 and the two Papua New Guinea isolates have been shown to be at least 7% divergent in other areas of the genome (NS1 and E). None of the changes found in the 5' non-coding region appreciably altered the predicted secondary structure. It is noteworthy that none of the flaviviruses examined to date possess the consensus sequence surrounding the initiator codon (GCC(A/G)CCAUGC which has been found in most eukaryotic mRNAs nor do they possess the in phase GCC unit upstream from AUG. The conservation of secondary structure among these isolates, and flaviviruses in general, suggests that structure rather than primary sequence may be important for the initiation of translation with these viruses.

RNA SYNTHESIS OF A POLIOVIRUS MUTANT IN VIVO AND IN VITRO

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3NC202, a temperature-sensitive mutant of poliovirus with an insertion in the 3' noncoding region, has its primary defect in RNA synthesis (Sarnow et. al., Proc. Natl. Acad. Sci. USA, 83:571-575, 1986; P. Sarnow, personal communication). To distinguish positive and negative strand synthesis, strand-specific probes were hybridized to RNA from 3NC202-infected cells. In comparison with control samples kept at the permissive condition of 32.5°C, cells shifted to 39.5°C several hours post-infection accumulated nearly normal amounts of positive strand RNA but little negative strand RNA. Thus, the primary defect of 3NC202 is in negative strand synthesis.

To study the specificity of poliovirus RNA replication in vitro, we tested whether 3NC202 negative strand synthesis in vitro was temperature sensitive. Our in vitro system used highly purified poliovirus RNA-dependent RNA polymerase, partially purified terminal uridylyl transferase (TUTase), and a virion RNA template. TUTase can act as host factor for negative strand synthesis in vitro by adding oligo(U) to the 3' end of the positive strand, allowing a hairpin primer to form. We quantified RNA synthesis at 32.5°C and 39.5°C from mutant and wild-type templates. Over a range of template concentrations, the behavior of 3NC202 was almost identical to that of wild type, with slightly less RNA synthesized from both templates at 39.5°C. Thus, this in vitro system does not mimic the specificity observed in vivo for 3NC202 RNA synthesis.

VARIANT FOOT-AND-MOUTH DISEASE VIRUS FROM PERSISTENTLY INFECTED CELLS.

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In the course of a persistent infection of BHK-21 cells with foot-and-mouth disease virus (FMDV) a coevolution of the cells and the resident virus occurs (de la Torre et al. J. Virol. 62, 2050-2058, 1988). We have cloned and sequenced the structural protein-coding region of the variant virus dominant after 100 passages of the carrier cultures. Several amino acid substitutions were fixed in VP1, VP2 and VP3, particularly at the N-terminus of VP3. The latter have been confirmed by direct protein sequencing of purified VP3. This variant virus shows multiple phenotypic alterations, including an increased ability to overcome a restriction imposed by the coevolved host cells.

HUMAN RHINOVIRUS SEROTYPE 2: IN VITRO SYNTHESIS OF AN INFECTIOUS RNA

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A strategy for the synthesis of a complete cDNA copy of the HRV2 genome has been developed; this cDNA copy was placed under the control of a 17 RNA polymerase promoter. An *in vitro* transcribed RNA containing two extra G residues at the 5' end gave rise to plaques on transfection into HeLa cells. The efficiency was approximately half that obtained with viral RNA. On the contrary, an *in vitro* synthesised RNA containing 16 additional nucleotides at the 5' end was not infectious. This ability to make an infectious *in vitro* transcribed RNA will be useful in studying virus-receptor interactions and other aspects of the virus life cycle. The use of site-directed mutagenesis to produce viruses with altered properties will be described.

STRUCTURAL ANALYSIS OF NODAMURA VIRUS RNA2, THE MESSENGER RNA FOR THE COAT PROTEIN PRECURSOR.

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Nodamura virus (NOV), originally isolated from mosquitoes, is the type member of the family of insect viruses called Nodaviridae. It is unique among the Nodaviridae in its ability to cause fatal infection in vertebrates as well as in insects. Moreover, NOV, unlike other closely related Nodaviruses, does not infect cultured *Drosophila* cells and does not show any cytopathic effect in most insect cell lines. Transfection of NOV RNAs into *Drosophila* cells shows that NOV viral RNA synthesis is not impaired. This suggests a change in the region of coat protein responsible for interaction with the host. We have, therefore, determined the nucleotide sequence of NOV RNA2 and compared it with the known sequence of black beetle virus (BBV) RNA2 [Dasgupta et al., Nucl. Acids Res. 12, 7215-7223 (1984)]. The deduced amino acid sequence was compared and mapped relative to the BBV structure obtained by refinement of X-ray crystallography data reported previously [Mosur et al., Proteins: Structure, function and genetics 2, 167-176 (1987)]. The results show an average of fifty percent difference in the primary sequence at both the nucleotide and amino acid level. The majority of the amino acid difference mapped near the outer surface of the BBV virion, a possible area for interaction with the cell surface.

We were interested in screening a series of isolates of the protozoan *Leishmania* for the presence of viruses. The experimental procedure we used was based on an enzymatic assay originally developed for viral RNA-dependent RNA polymerases. Simultaneously, total promastigote nucleic acid preparations were analyzed for the presence of viral genome and/or transcripts. Two isolates, both classified as *L. braziliensis guyanensis*, were found to be positive for RNA polymerase activity and to carry a large (5kilobases) RNA species. The polymerase reaction products hybridized to the 6 kb RNA, believed to be the viral genome. In conjunction with electron microscopical observations these results indicate the presence of an RNA virus in these *Leishmania* isolates. Preliminary evidence suggests that in our RNA dependent RNA polymerase assay we are making primarily plus sense RNA. Although we are not certain of the polarity of our virus, given the size of the genome and the other evidence we think we are looking at a new plus sense RNA virus.

NUCLEOTIDE SEQUENCE AND GENOME ORGANIZATION OF BROAD BEAN MOTTLE VIRUS; SEQUENCE HOMOLOGIES WITH OTHER BROMOVIRUSES.

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In order to study virus-encoded functions which are involved in virulence, host range and other plant-virus interactions, we do interrelated and comparative studies of three bromoviruses: broad mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV) and broad bean mottle virus (BBMV).

In this communication we present the complete sequence and genome organization of BBMV. Like in BMV and CCMV, the BBMV genome is composed of four single-stranded positive-sense components. The length of BBMV RNAs 1 and 2 is similar to that for BMV and CCMV, whereas BBMV RNA3 has longer 5' end noncoding region and longer intergenic region. The RNAs are capped at the 5' end and contain a tRNA-like structure at the 3' end. Unlike for BMV, CCMV and other similar viruses BBMV subgenomic RNA4 has an A as its 5' terminal nucleotide suggesting some special features in the mechanism of replication of BBMV RNAs.

Sequence analysis indicate that BBMV RNAs 1 and 2 are monocistronic whereas RNA3 is dicistronic. These data agree with the results of *in vitro* translation experiments.

Sequence homologies among analogous RNA components comprise most of the length of RNA1 and 2 in three bromoviruses. On the other hand, components 3 differ in sequence substantially.

TYMOVIRUSES : STRATEGIES OF EXPRESSION AND INSERTION IN THE "SINDBIS-LIKE" SUPERGROUP.

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Tymoviruses are icosahedral plant RNA viruses with a monopartite positive stranded genome of about 6000 nucleotides. The type-member is Turnip Yellow Mosaic Virus (TYMV). The interest raised by TYMV mostly relies on the tRNA-like properties of its genome (not discussed here) and on the variety of strategies it utilizes to express as many as 9 proteins from a unique and relatively small genomic RNA. Recent translation experiments will be presented that characterize the proteolytic processing step involved in the synthesis of some of its nonstructural proteins.

Molecular cloning and sequencing of the genomes of TYMV (1) and of several other tymoviruses confer to these viruses the status of appropriate experimental models to study viral replication, host specificity and to test anti-viral strategies such as the "sense-RNA" approach (2).

Finally sequence comparisons among the non structural proteins encoded by tymoviruses and by other plant and animal (+) RNA viruses stress the conservation of the already described NTP-binding and polymerase domains. All these observations lead to the insertion of the tymovirus group within the "Sindbis-like" supergroup of (+) RNA viruses as opposed to the "picorna-like" supergroup and lend support to the hypothesis of modular evolution for these viruses.

1. Morch, M.D., Boyer, J.C. and A.L. Haenni (1988) Nucl. Acids Res. 16:6157-6173.

2. Morch, M.D., Joshi, R.L., Denial, T.M. and Haenni, A.L. (1987) Nucl. Acids. Res. 15:4123-4130

SYNTHESIS OF TURNIP YELLOW MOSAIC VIRUS SUBGENOMIC RNA IN VIVO AND IN VITRO : COMPARISON WITH ALPHAVIRUSES.

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Turnip Yellow Mosaic Virus (TYMV) possesses a single-stranded (+) RNA genome. This genomic RNA is polycistronic, the coat protein gene being 3' proximal. The coat protein is synthesized via a subgenomic RNA.

To investigate the mechanism leading to the formation of the subgenomic RNA *in vivo*, the double-stranded RNAs isolated from infected leaves were characterized by Northern blot hybridization under native and denaturing conditions and by direct labelling of nascent (+) RNA strands. The results obtained demonstrate that the subgenomic RNA is synthesized *in vivo* by internal initiation of replication on a (-) RNA of genomic size.

Replication experiments were performed *in vitro* to identify on the (-) RNA the internal promoter involved in this mechanism. Our results indicate that the "core" of this promoter is located 3' of the sequence corresponding to the subgenomic RNA, at a distance of 26 to 55 nucleotides from the start of replication of this RNA. Furthermore the nucleotide sequence in this promoter region shares homology with the consensus sequence of the internal promoter determined for animal alphaviruses.

DENGUE VIRUS EPIDEMIOLOGY DETERMINED BY LIMITED GENOMIC SEQUENCING

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Dengue viruses have progressively extended their geographic distribution and are now the most important mosquito-borne viruses associated with human illness. Determining the genetic variability and transmission patterns of these viruses is crucial in developing effective control strategies for the disease. Eighty geographically and temporally diverse dengue virus strains of human and mosquito origin were compared by primer-extension sequencing of the RNA template. Forty isolates represent serotype 1 and the remainder serotype 2. Comparison of nucleotide sequences from 3% of the dengue genome provided sufficient information for determining genetic relationships among these virus isolates. The analysis of 240 nucleotides from the E/NS1 gene region revealed that the evolutionary patterns of dengue viruses of serotypes 1 and 2 are different, as are the transmission pathways of the viruses across the world. The quantitative comparison of these nucleotide sequences disclosed previously unknown evolutionary relationships between disease outbreaks. Viruses fell into five distinct genotypic groups for each of the two serotypes. Maximum divergence across the E/NS1 gene region among type 1 virus isolates reached 9%, while for type 2 strains it was almost twice as high. For type 2 viruses, one genotypic group represents an isolated, sylvatic virus cycle which apparently has evolved independently in Africa. This is the first genetic evidence that a sylvatic cycle of dengue virus exists.

INFECTIOUS THEILER'S VIRUS cDNA CLONES: STUDIES OF INTRATYPIC RECOMBINANTS AND VIRAL POLYPROTEIN PROCESSING

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Theiler's murine encephalomyelitis viruses (TMEV) are mouse picornaviruses that can be separated into two subgroups on the basis of their biological activities. DA strain and other members of the TO subgroup cause a persistent demyelinating infection in mice, while GDVII strain and other GDVII subgroup strains cause an acute lethal polioencephalomyelitis and neither persist nor demyelinate. We generated an infectious DA clone inserted into a transcription vector. The infectivity of *in vitro* derived DA transcripts was enhanced by reengineering the clone to bring the 5' end of the DA genome two nucleotides downstream from the T7 promoter. Virus derived from transfection of the transcripts produced an inflammatory demyelinating disease in mice indistinguishable from DA wild type virus. Using the DA infectious clone and GDVII cDNA subgenomic clones, we were able to produce chimeric genomes and interstrain recombinant viruses that will help to identify the genetic loci responsible for the strains' varied biological activities.

In order to investigate polyprotein processing of Theiler's murine encephalomyelitis viruses (TMEV), we analyzed *in vitro* translation reactions programmed by *in vitro* derived transcripts from the infectious full-length DA cDNA clone. To help identify the proteinases that carried out the processing, the DA cDNA clone transcription template was modified by linearization with varied restriction endonucleases that cut the template at different lengths or by linker insertion and/or deletion mutations in putative proteinase-coding regions. This information may be important in further investigations of the abnormal virus expression seen in DA virus late demyelinating disease, since polyprotein processing is critical in determining picornaviral gene expression.

RAPID MOLECULAR EVOLUTION OF WILD TYPE 3 POLIOVIRUS INFECTION OF INDIVIDUAL HOSTS, LEENA KINNUNEN¹, ANITA HUOVILAINEN and TAPANI HOVI² Molecular Biology Unit National Public Health Institute, Helsinki, Finland

The mutation rate of RNA viruses is known to be high, which allows for great adaptability and rapid evolution in presence of selection mechanisms.

An outbreak of poliomyelitis with widespread circulation of wild type poliovirus 3 throughout the country was discovered in Finland between August 1984 and January 1985. Finland had been free of poliomyelitis since 1964 as a result of a high coverage immunization programme with the inactivated poliovirus vaccine.

In this study the extent of molecular variation and evolution was followed during wild type poliovirus 3 replication in several individuals. Altogether, the antigenic characteristics of 153 plaque purified virus strains from sequential faecal specimens from eight patients were analyzed with a pattern of nine monoclonal antibodies. Selected plaque purified viruses (34 strains) were further studied by partial RNA sequencing. The sequenced regions encode amino acids that are exposed on the virus surface and constitute the major antigenic sites. Almost every clinical isolate seemed to be a mixture of variants. Very often rapid evolution took place between sequential isolates and amino acid substitutions were seen at the known antigenic sites.

HOMOLOGOUS POTY-, FLAVI- AND PESTIVIRUS PROTEINS BELONGING TO A SUPERFAMILY OF HELICASE-LIKE PROTEINS. RNA STIMULATED ATPase ACTIVITY OF PLUM POX POTYVIRUS CI PROTEIN.

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Plum pox potyvirus genome consists of a 9786 nucleotide long positive sense RNA molecule with its 5' end covalently linked to a protein (VPg) and a 15-500 nucleotide long polyA tail at its 3' end. An AUG triplet at position 147-149 has been assigned as the initiation codon for the translation of a genome size viral polyprotein of 3140 amino acid residues. The nucleotide sequence of the non-coding regions and the predicted amino acid sequence of the polyprotein were compared with those previously reported for other potyviruses as well as with other sequences from viral or cellular origin. Cleavage of the PPV polyprotein at several of the putative recognition sequences for the 49kD PPV protease has been confirmed by amino terminal sequencing of the processed products and site directed mutagenesis. The potyviral CI protein, which contains sites A and B of the so-called NTP-binding motif, is closely related to the NTP motif-containing proteins of animal flavi- and pestiviruses and to a superfamily of cellular helicase-like proteins. Preliminary results indicate that the CI protein of plum pox virus presents *in vitro* ATPase activity stimulated by the presence of RNA.

REPLICATIVE FORM OF NUDAURELIA β VIRUS RNA

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Nudaurelia β virus (NBV), which infects the emperor moth, *Nudaurelia cytherea capensis*, is the type member of the Tetraviridae, a recently established family of insect-pathogenic small riboviruses with T=4 icosahedral capsid symmetry. A cell line supporting the replication of these viruses has yet to be found. Thus, little is known of their replication strategy, except that cell-free translation of the genomic RNA results in the synthesis of a range of polypeptides, none of which corresponds to the single coat protein of M_r about 65000.

The genome of NBV consists of a single strand of positive-sense RNA of about 5.3 kilobases. Extraction of double-stranded RNA (dsRNA) from individual virus-diseased larvae yielded only one major species with twice the M_r of the genomic RNA. This dsRNA hybridized with a radio-labelled virion RNA probe, and is thus probably the replicative form of the viral genome. No other dsRNAs were detected unless the agarose gels were overloaded with material extracted from pooled larvae. None of these minor dsRNAs had sequences in common with the genomic RNA probe. No dsRNAs could be detected in larvae that were virus-free.

None of the polypeptides produced by cell-free translation of the virion RNA were precipitated by a polyclonal anti-NBV serum. In the absence of evidence for a subgenomic RNA, the mechanism of coat protein synthesis remains obscure.

CIS-ACTING REGULATORY SEQUENCES IN ALPHAVIRUS GENOMES
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Comparative sequencing of alphavirus genomes has identified four regions which exhibit a high degree of nucleotide conservation. It has been postulated that these sequences serve as replicase binding sites. A full-length cDNA clone of Sindbis virus, the type alphavirus, has been utilized in a molecular genetic approach to study the function(s) of these regions. In particular, the 3' 19 nucleotide conserved region along with the genomically encoded poly(A) tract has been analyzed by site-directed mutagenesis to determine the role of such cis-acting sequences in minus-strand RNA synthesis.

We report here the construction of a full-length cDNA clone from which infectious RNA can be transcribed of Ross River virus, another member of the alphavirus genus. This has enabled us to construct hybrid viruses between Ross River and Sindbis. In particular, the 5' and 3' non-translated regions of each virus have been exchanged and the effect on virus replication has been studied. In addition we have generated a hybrid virus which contains the nonstructural proteins of Sindbis and the structural proteins of Ross River. This virus displays a host range similar to Ross River virus and a replication efficiency intermediate between Sindbis and Ross River. This virus, and other structural protein hybrids, will prove useful in investigating the pathogenic properties exhibited by these viruses.

ORGANIZATION OF BEET YELLOW CLOSTEROVIRUS GENOME

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Virion RNA of beet yellows virus (BYV) is a messenger-sense RNA of about 14.5 kilobases containing no poly(A). Its 3' end is represented by the non-coding sequence sharing no obvious similarity with the tRNA-like structures in plant viral RNA genomes reported so far. Translation of BYV RNA in cell-free system yielded a major polypeptide of 250K and some lighter products from which 66K was the most prominent. None of the products was found to coincide with the coat protein in electrophoretic mobility or immunological properties. The synthesis of all the BYV RNA-directed polypeptides was blocked by the cap analogue, thus suggesting the presence of a cap structure at the 5' end of virion RNA.

The single-stranded RNA from BYV-infected plants contained at least six RNA species of genomic and subgenomic (6.3, 4.8, 2.7, 1.6, and 1.0) sizes. Double-stranded analogues have been found for all these RNAs. Thus, the strategy of BYV genome expression possibly involves formation of subgenomic RNAs.

BYV displays sharp differences with another representative of closterovirus group, apple chlorotic leaf spot virus, in genome size and the absence of poly(A). Hence these viruses can be hardly grouped together.

THE MAJOR ECHO VIRUS SUBGROUP IS GENETICALLY CLOSELY RELATED TO COXSACKIE B VIRUSES

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ECHO viruses are the largest subgroup of enteroviruses comprising 31 serologically distinct members. Comparative analysis by using nucleic acid hybridization has revealed that a majority of ECHO viruses resemble coxsackie B viruses (Auvinen et al., Arch. Virol., in press). ECHO virus 22, however, is an exception and does not share homology with any of the enteroviruses studied. In order to understand the organization of ECHO virus genomes at a detailed level we have started nucleotide sequence analysis of ECHO viruses 11 and 22. The electron microscopic appearance and the genome length of both ECHO virus 11 and 22 equal to coxsackieviruses. The nucleotide sequence homology of ECHO virus 11 at the P3 region is approximately 80 % when compared to coxsackievirus B3. The proteolytic cleavage sites studied so far are identical with those found in other related enteroviruses. The organization of the 3' noncoding region of ECHO virus 11 is also equal to coxsackie B viruses but different from polioviruses. On the other hand, the preliminary 3' end sequence of ECHO virus 22 genome does not show any significant homology with other members of human enteroviruses. An oligonucleotide derived from the ECHO 22 virus sequence hybridizes exclusively with this strain.

INDICATIONS FOR A PSEUDOKNOT STRUCTURE IN THE RNA GENOME OF PLRV

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Potato leafroll virus (PLRV) is a member of the luteovirus group. The plus-sense single-stranded RNA genomes of luteoviruses are contained within icosahedrally shaped virions. These are only infectious when applied via aphids. Therefore, PLRV infections give rise to considerable losses in potato yields when many aphids are present.

The PLRV genomic RNA encodes six putative gene products in open reading frames (ORFs) 1 to 6. For expression of these 6 ORFs the virus might be using three different methods. ORF1 starts with a suboptimal AUG according to the Kozak rules. This way ORF2 can be expressed from the genomic RNA as well. ORF3 seems to be expressed via a -1 translational frame-shift in ORF2. In the region where frameshifting might occur a possible pseudoknotted structure can be found. A similar feature can be observed in the equivalent region of the genome of BWYV. This might have a function in ribosomal frameshifting. ORF3 bears a 43% homology with the putative RNA-dependent RNA polymerase of southern bean mosaic virus. The expression of ORFs 4 to 6 most likely is via a subgenomic messenger of 3.4 kb found in infected tissue. ORFs 4 and 6 are contiguous reading frames separated only by an UAG (amber) stop codon. ORF4 encodes a protein showing homology with other luteovirus coat proteins. ORF5, which is contained entirely within ORF4, might encode a precursor of the VPg molecule.

HOG CHOLERA VIRUS - CHARACTERIZATION OF SPECIFIC ANTISERUM AND IDENTIFICATION OF cDNA CLONES

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A specific antiserum was raised against the pestivirus inducing hog cholera (hog cholera virus, HCV). Using immunoprecipitation and SDS-PAGE, this antiserum served for comparison of HCV-induced proteins with those from a related and better characterized pestivirus, bovine viral diarrhoea virus (BVDV). In addition to immunological relationships, the apparent molecular weights of some proteins induced by both viruses were quite similar.

HCV genomic RNA was found to be about 12 kb in length, comparable to BVDV RNA. cDNA was synthesized starting from RNA isolated from partially purified virions and cloned in lambda-gt11. Screening with the antiserum resulted in identification of several positive clones. Partial sequencing of one HCV-derived cDNA clone revealed a high degree of homology to a portion of the BVDV sequence.

TEMPERATURE-SENSITIVE SHUTOFF OF ALPHAVIRUS MINUS STRAND SYNTHESIS MAPS TO NSP4.

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Alphavirus minus strand synthesis occurs only early in infection and is coupled to synthesis of viral nonstructural proteins and to formation of new viral replication complexes. Our previous results identified a mutant (ts24 of the A Complementation group) of Sindbis virus (SIN HR) that failed to cease minus strand synthesis late in infection in the absence of new protein synthesis if infected cells were shifted to 40°C (Sawicki and Sawicki, Virology 151:339, 1986). Revertants of ts24 (ts24R) retained this ts phenotype, indicating the defect in temporal regulation of minus strand synthesis was not conditionally lethal and could map outside the A cistron. Minus strand synthesis by ts24R apparently occurred in previously formed replication complexes that had been engaged in plus strand synthesis. The infectious clone of SIN HR, Toto1101, was used to identify the region of the genome of ts24R responsible for this phenotype. Three specific cDNAs that together represented the entire genome of ts24R were exchanged for their corresponding regions in Toto1101 and infectious transcripts used to prepare hybrid viruses. The phenotype of ts24R was present in the region nt 2288-nt 7999, encompassing part of nsP2-nsP3-nsP4 encoding sequences. Subcloning and sequencing identified a single nucleotide change at nt 6339 (C to A, predicting a Gln to Lys change at aa 195 in nsP4) that was common among but unique to ts24 and its revertants. Nucleotide changes at the 5' and 3' ends of the ts24R genome did not affect minus strand synthesis. Substitution of the wildtype nucleotide at position 6339 in an infectious clone of ts24R should eliminate the ts24R phenotype. We conclude that ts failure to cease minus strand synthesis by ts24 and its revertants maps to the nsP4 region.

NUCLEOTIDE SEQUENCE OF THE GENOME AND COMPLETE AMINO ACID SEQUENCE OF THE POLYPROTEIN OF THE TICK-BORNE ENCEPHALITIS VIRUS.

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We have now cloned and sequenced the genomic RNA of tick-borne encephalitis virus (TBEV) that encodes all structural and nonstructural proteins. The complete genome is 10,480 bases in length with a single open reading frame extending from nucleotides 127 to 10,365 encoding 3,417 amino acids. The 5'- and 3'-noncoding extremities present stem- and loop-structures. A polyprotein precursor is apparently proteolytically cleaved by a mechanism resembling that proposed for expression of polyproteins of other flaviviruses such as Yellow fever, West Nile and Kunjin viruses. The deduced TBEV gene order is 5'-C-pr(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. The genome and the polyprotein of TBEV and other flaviviruses are transmitted to their vertebrate hosts by different vectors such as ticks and mosquitoes. Comparison of sequence homology of polyproteins suggests that TBEV is more closely related to Y. fever virus than to flaviviruses of other serological subgroups (West Nile or Dengue viruses). The hydrophobicity profiles of the flavivirus polyproteins are highly conserved. Nonstructural proteins NS2A, NS2B, NS4A, and NS4B are extremely hydrophobic, suggesting that these proteins are likely associated with cellular membranes. Proteins E, NS1, NS3, and NS5 are the most conservative and may be involved in general activities related to viral replication.

EFFECT OF ACTINOMYCIN D ON REPLICATION OF SATELLITE TOBACCO RINGSPOT VIRUS RNA IN PLANT PROTOPLASTS.

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The 359 nt satellite tobacco ringspot virus RNA (sTobRV RNA) is a molecular parasite of its supporting virus, tobacco ringspot virus (TobRV). That is, sTobRV RNA requires co-infection with TobRV for its propagation, it reduces the yield of TobRV, and it becomes encapsidated in TobRV coat protein. Specific contributions of TobRV gene products, other than coat protein, to the life cycle of sTobRV RNA are unknown. We have been studying sTobRV RNA replication in this three-component system of host, virus and satellite RNA. DNA-dependent RNA synthesis was effectively inhibited in cowpea (*Vigna unguiculata*) leaf protoplasts incubated in a medium containing 50 µg/ml actinomycin D (Act D), as measured by incorporation of [¹⁴C]uridine into RNA. When this concentration of Act D was added to protoplasts 24 hr prior to co-inoculation with sTobRV RNA and TobRV genomic RNAs, no accumulation of sTobRV RNA was detected by blot hybridization after electrophoresis of RNA preparations. This apparent inhibition of sTobRV RNA synthesis did not appear to be due to interference with transcription, since additions of Act D at 24 hr or 48 hr after co-inoculation resulted in only slightly weaker sTobRV RNA signals than those obtained with no addition of Act D. Our results and previous findings of RNA complementary to encapsidated satellite RNA in extracts of infected tissue imply that an RNA-dependent RNA polymerase is responsible for the synthesis of sTobRV RNA. The strongly inhibitory effect of Act D, added early, on sTobRV RNA synthesis suggests a role for a host factor in the early phase of sTobRV RNA replication.

MAPPING OF BROMOVIRUS RNA REPLICATION FUNCTIONS BY CONSTRUCTION OF HYBRID RNA2 MOLECULES. Patricia L. Traynor* and Paul G. Ahlquist. Institute for Molecular Virology, University of Wisconsin-Madison, Madison, WI 53706, USA.

The bromovirus group of plant viruses has a tripartite (+)-sense RNA genome similar in sequence and organization to a wide range of other plant viruses. Replication of bromo mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) requires both RNAs 1 and 2 and the 1a and 2a proteins they encode, respectively. Despite extensive homology between bromoviral RNA2 nucleotide and peptide sequences, several functional differences distinguish RNA2 and the 2a protein of BMV from those of CCMV. First, successful infection of barley protoplasts requires a virus-specific compatibility between RNA2 and its homologous RNA1, since heterologous combinations of RNAs 1 and 2 do not support viral RNA replication. Secondly, although BMV RNA2 is amplified by CCMV, RNA2 from CCMV is not amplified by BMV, indicating a difference in template activity between the two RNA2s. Construction of hybrid RNA2 molecules containing various portions of BMV and CCMV sequences has allowed preliminary mapping of these functional differences. Additionally, hybrid RNA2 molecules that are compatible with RNA1 from either virus have been used to show that a virus-specific difference in the amplification of RNA3 templates appears to segregate with RNA1.

GENOMIC AND ANTIGENIC COMPARISONS OF EASTERN EQUINE ENCEPHALITIS VIRUSES AND RELEVANCE TO VIRAL EVOLUTION. Patricia M. Repik* and Julie M. Strizki. Department of Microbiology and Immunology, The Medical College of Pennsylvania, Philadelphia, PA, U.S.A.

To investigate the genetic and antigenic diversity of both North and South American EEE viruses, the RNAs of more than 20 isolates were analyzed by RNA fingerprinting, and the virion proteins were characterized by PAGE and Western blot analysis. All the N. Am. isolates displayed strikingly similar fingerprint patterns, with 72-98% oligonucleotide homology. The strong genetic relationship among these strains was stable with time, host species, and geographic distribution. Conversely, the S. Am. isolates displayed fingerprint patterns which differed markedly from the N. Am. strains and, in addition, were much more diverse amongst one another. Their oligonucleotide homologies ranged from 17-92%. Both geographic distribution and time appeared to influence the genetic relatedness of the S. Am. strains. Analysis of viral proteins supported these data in that minor variation was generally observed only in the E2 protein of the N. Am. strains, whereas more extensive variation in both the E1 and E2 proteins were seen among the S. Am. strains. Although the North American and South American EEE strains differed genetically and antigenically, major immunogenic epitopes were preserved as demonstrated by Western blot analysis. The possibility that selective pressures exerted by insect vectors may play an integral role in the evolution of EEE viruses is intriguing.

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF THE GENOME OF HOG CHOLERA VIRUS
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A cDNA clone derived from genomic RNA of hog cholera virus (HCV) was identified using an oligonucleotide complementary to the RNA encoding a hexapeptide from the putative RNA dependent RNA polymerase of the closely related bovine viral diarrhoea virus (BVDV). This clone served as a probe for screening different size selected cDNA libraries. After molecular cloning and nucleotide sequencing the HCV genome was shown to consist of 12284 nucleotides containing one long open reading frame. Sequence comparison revealed a high degree of homology between HCV and BVDV genomic RNAs. With respect to HCV the genome of BVDV contains an insertion coding for 90 amino acids.

DESCRIPTION OF THE SEQUENCE OF HOG CHOLERA VIRUS RNA: COMPARISON OF THIS SEQUENCE WITH THAT OF BOVINE VIRAL DIARRHOEA VIRUS.
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Hog cholera virus-specific RNA, synthesized in infected SK-6 cells (Moormann and Hulst, 1988, Virus Res., 281-291), was cloned and sequenced. Various cDNA libraries were prepared to completely clone the virus-specific polyA minus RNA. The first strand of the cDNA was primed with either oligonucleotides prepared from calf thymus DNA, or oligo-dT on polyA adenylated RNA, or specific oligonucleotides deduced from the sequence.

The cloned sequence is 12,280 nucleotides in length. The sequence of several independent clones primed with oligo-dT suggests that the 3'-end of the viral genome is completely cloned. Whether the sequence is also complete at the 5'-end is still being investigated.

One large open reading frame (ORF), encoding a polyprotein sequence of 3897 residues, was found in the second reading frame of the sequence of one of the cDNA strands. The sequence of this strand corresponds with that of the viral plus strand RNA.

The amino-acid sequence encoded by the ORF will be described and the sequences of hog cholera virus and bovine viral diarrhoea virus (members of the Pestivirus genus) will be compared.

CHARACTERISTICS AND EFFECTS OF DEFECTIVE INTERFERING PARTICLES IN HEPATITIS A INFECTED CELLS

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Adaptation of hepatitis A virus to cell culture usually leads to the establishment of persistent infection. Such a virus/cell relationship might be favored by the presence of defective interfering particles (DIP). Indeed, DIP with distinct deletions in the genome could be demonstrated for various HAV isolates in several cell culture systems and at different *in vitro* passage levels. To test at which level DI particles interfere with replication of standard HAV virions, cultures were infected at low and high m.o.i. and with virus pools established at early (11th) and late (44th) *in vitro* passage levels. Subsequently, production and excretion of viral antigen, infectious particles, and temperature permissive mutants, as well as synthesis and accumulation of vRNAs during the replication cycle of HAV was analysed. The quantity of genomic vRNA oscillated throughout the period of observation. Defective RNAs could always be demonstrated, but peak amounts usually were present after genomic RNA reached maximum concentrations. Presence of DIP evidently interfere with synthesis of vRNA and the production of progeny virus particles. Production of viral antigen, however, seemed to be unaffected.

HIGH-FREQUENCY LEADER SEQUENCE SWITCHING DURING CORONAVIRUS DI RNA REPLICATION.

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A system was developed exploiting defective-interfering (DI) RNAs of coronavirus to study the role of free leader RNA in RNA replication. A cDNA copy of mouse hepatitis virus DI RNA was placed downstream of 17 RNA polymerase promoter to generate DI RNAs capable of extremely efficient replication in the presence of a helper virus. We demonstrated that, in the DI RNA-transfected cells, the leader sequence of these DI RNAs was switched to that of the helper virus during one round of replication. This high-frequency leader sequence exchange was not observed if a nine-nucleotide stretch at the junction between the leader and the remaining DI sequence was deleted. This observation suggests a novel discontinuous replication of an RNA with uninterrupted sequence.

THE SYNTHESIS, MODIFICATION AND SECRETION OF TBEV GLYCOPROTEINS.

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The accurate and efficient production of cellular or viral proteins, by native and genetically engineered systems depends on molecular signals for the correct folding, post-translational modification and intracellular transport of those proteins. The glycoproteins of TBE virus, preM, E and NS1 offer an excellent system to study the intracellular transport of proteins. E is present in virus particles, but not on the plasma membrane; preM is present only in infected cells and its non-glycosylated product; M is only found in virus particles, and NS1 is never found in virions, but is secreted from the cell and has been detected on the plasma membrane. This situation not only enables a study of protein transport, but as all viral proteins are translated from a single message, gives the virus some unique problems in the control of protein synthesis.

Work in our laboratory has demonstrated that the synthesis of these proteins is strictly controlled and is dependent upon the subcellular environment of the translation machinery. It is also apparent, that at least for NS1, the level of glycosylation reflects the destiny of the protein. The effect of sorting sequences, glycosylation and protein folding on subcellular transport will be discussed in this presentation.

CHANGES IN MOLECULAR STRUCTURE AND MEMBRANE ASSOCIATION OF THE DENGUE-2 VIRUS PROTEIN NS1 DURING MATURATION AND TRANSPORT

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The dengue-2 virus nonstructural protein NS1 is a glycosylated, acidic protein with an approximate mol. wt. of 46000. For a short time after translation NS1 appears as a monomeric, water soluble protein. Within 20-40 min after synthesis the protein forms homodimers which show increased hydrophobicity as indicated by means of Triton X-114 phase partitioning. This dimeric form of NS1, in contrast to the monomeric NS1, is found in the membrane fraction of the cell homogenate suggesting that dimerization and membrane association are closely correlated.

The NS1 dimers are transported through the Golgi system where - in mammalian cells - two of the four asparagine linked carbohydrates are processed to complex glycans.

About 60-80 min after translation NS1 is secreted into the medium where it is found in both a pelletable and soluble form. Both forms are protein dimers but show differences with respect to molecular organization as shown by immunogold electron microscopy. The data are consistent with the hypothesis that pelletable extracellular NS1 is associated with membrane vesicles.

EXPRESSION OF COWPEA MOSAIC VIRUS M RNA IN COWPEA PROTOPLASTS.

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In cell free systems cowpea mosaic virus (CPMV) M RNA is translated into two polyproteins, 105K and 95K. Using antiserum against the small capsid protein VP23, these proteins have now been detected in cowpea protoplasts, a few hours after inoculation with CPMV. Also at later stages of infection these proteins could be detected, but only if proteolytic processing was inhibited by the addition of ZnCl₂. Using antiserum against a synthetic peptide, corresponding with a part of the overlapping C-terminal ends of the 58K and 48K proteins, the 58K protein, being the amino-terminal cleavage product of the 105K protein, was found in the cytoplasmic fraction of infected protoplasts, whereas the 48K protein, derived from the 95K protein, was detected in both the cytoplasmic and membrane fraction of protoplasts. The presence of the 105K, 95K, 58K and 48K proteins in CPMV-infected protoplasts indicates that, similar what has been found *in vitro*, also *in vivo* distinct initiation codons on the M RNA are used to produce the 105K and 95K polyproteins.

SYNTHETIC PEPTIDE ANTIBODIES AGAINST HUMAN RHINOVIRUS TYPE 14 PROTEASE 3C.

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Human rhinoviruses (HRVs), which form one genus of the family Picornaviridae, are the main causative agents of the common cold. The basic organization and replication of the positive strand RNA genomes (7.5-8.0 kb) of Picornaviruses, e.g. HRVs, poliovirus and foot-and-mouth disease virus, are similar. The primary translation product of Picornaviruses is a single precursor polypeptide which is processed primarily by viral protease 3C to mature products. Although the primary amino acid sequence of 3C proteases has diverged considerably, there are short stretches of highly conserved amino acids. We have synthesized two synthetic peptides of 16 and 11 amino acids based on two of the highly conserved amino acid sequences in protease 3C, and raised peptide-specific antibodies in rabbits. The specificities of the peptide antibodies for protease 3C were verified using dot blot and ELISA assays. Immunoprecipitation experiments demonstrated that both the peptide antibodies recognized the protease 3C expressed by HRV-14 recombinant plasmid pKCC110 in *E. coli* maxicells. Experiments are in progress to determine whether these antibodies neutralize the biological activity of protease 3C.

CHARACTERIZATION OF SEMLIKI FOREST VIRUS SPIKE GLYCOPROTEINS DEFICIENT IN p62 CLEAVAGE

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The subgenomic 26S cDNA coding for the structural proteins of Semliki Forest virus (SFV), an alphavirus, was expressed via a recombinant vaccinia virus vector in BHK cells. The SFV polyprotein precursor is correctly cleaved and processed as judged from PAGE profiles, and transported to the plasma membrane.

We have generated specific mutations in the structural glycoproteins of SFV and we are using the vaccinia virus expression system to study their phenotype effects on the assembly of this simple enveloped RNA virus. The membrane of the SFV particle contains 240 copies of the spike heterodimer E1/E2. The E2 spike glycoprotein originates from the precursor protein p62 which is cleaved late in virus maturation, prior to arriving at the plasma membrane by a host enzyme recognizing dibasic residues. It has been proposed that the cleavage of p62 is a crucial event in triggering the budding of SFV.

Here we describe the phenotypes of SFV spike glycoproteins deficient in the cleavage of p62 expressed in BHK cells which were infected with the recombinant vaccinia virus vectors. Cell surface expression, polykaryon formation and E1/E2 oligomerization are addressed.

THE SEMLIKI FOREST VIRUS EMPLOYS MULTIPLE INTERNAL SIGNAL PEPTIDE CLEAVAGES TO GENERATE ITS MEMBRANE PROTEINS

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We are studying the mechanisms guiding the biogenesis of the Semliki Forest virus (SFV) membrane. The SFV is composed of a nucleocapsid structure surrounded by a membrane carrying the virus-specific glycoprotein spikes. The precursor polypeptide for the structural proteins is synthesized in the cytoplasm and is, after removal of the capsid protein (C) by self-cleavage, targeted to the ER membrane where it is translocated and co-translationally cleaved to yield the proteins p62, 6K and E1. In ER the p62 and E1 proteins form heterodimers preceding, and as a prerequisite to, their transport to the cell surface. The role of the 6K protein is still remains unclear. We have in detail characterized the nature of the various cleavages and topogenic signals which guide the correct generation of these transmembrane proteins. Our results reveal an astonishing scenario where internally alternating signal peptides and stop-transfer sequences of the polyprotein are used, through the action of the signal peptidase, to generate the structural proteins of the virus. Interestingly, none of the signal peptides are removed after cleavage, but remain as integral parts of the mature proteins, reflecting their additional roles in the assembly process of the virus.

MEMBRANE PROTEIN OLIGMERIZATION AND OLIGOMER DISSOCIATION AS POSSIBLE REGULATORS OF SEMLIKI FOREST VIRUS BUDDING AND FUSION

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The budding and the fusion processes of the enveloped animal virus Semliki Forest virus serve the purpose of transporting its nucleocapsid, containing its genome, from the cytoplasm of an infected cell into that of an uninfected one. We show here that, in the infected cell, the viral membrane (spike) proteins p62 and E1 are organized as heterodimers which are very resistant to dissociation in acidic conditions. In contrast, the mature form of the heterodimer, E2E1, which is found in the virus particle and which is generated by proteolytic processing of p62, is very prone to dissociate upon treatment with mildly acidic buffers. We suggest that this difference in behaviour of the intracellular precursor form and the mature form of the spike protein complex represents an important regulatory mechanism for the processes involving membrane binding around the nucleocapsid during budding and membrane release from the nucleocapsid at the stage of virus fusion.

EPITOPE MODEL OF TICK-BORNE ENCEPHALITIS-VIRUS ENVELOPE GLYCOPROTEIN E, ROLE OF CARBOHYDRATE SIDE CHAINS IN ITS ANTIGENIC STRUCTURE AND CONFORMATIONAL CHANGE OCCURRING AT ACIDIC pH. F. Guitrakho, F.X. Heinz, Ch. Kunz. Institute of Virology, University of Vienna, Vienna, Austria.

A large panel of monoclonal antibodies (MAbs) was made to characterize the antigenic sites of the Tick borne encephalitis (TBE) virus glycoprotein E. Nineteen epitopes were identified which differ with respect to serological specificity, functional activity or competitive binding of MAbs. Except three isolated epitopes i1, i2, and i3 these cluster to form three non overlapping domains termed A, B, and C. The structural properties of epitopes were assessed by analyzing the effect of chemical modifications (SDS-denaturation, reduction and carboxymethylation, performic acid oxidation, pH 5.0, CNBr and trypsin cleavage) on the antigenic reactivities of each epitope. Three epitopes of the domain A as well as i2 were SDS sensitive whereas all others were SDS resistant. Reduction and carboxymethylation abolished the antigenic reactivities of all epitopes of the domain B and also two SDS resistant epitopes of the domain A, indicating the role of disulfide bridges in stabilizing the conformation of these epitopes. Epitopes of the domain B could be localized on a 9000 Dalton trypsin fragment whereas the domain C could be identified on CNBr cleavage products.

Deglycosylation experiments using N-Glycanase^R resulted in destabilizing the carbohydrate containing C-domain so that the epitopes of this domain were no longer resistant to SDS denaturation or reduction by 2-mercaptoethanol.

A conformational change induced by low pH was revealed by differences of protease (trypsin and proteinase-K) cleavage maps. The conformational change, which involved the epitopes of domain A, i1 and i2 occurred between pH 6.0 and 5.5 with the threshold at pH 5.5.

STRUCTURE OF THE SINDBIS VIRUS NUCLEOCAPSID Angel Paredes, Kevin Coombs, Dennis T. Brown* The University of Texas at Austin 78713-7640 The Cell Research Institute

The topological arrangement of RNA and protein in purified Sindbis nucleocapsids has been examined by chemical, physical and morphological procedures. Chemical crosslinking experiments indicate that capsid protein exists in two conformations within the nucleocapsid. These conformations differ from that of free capsid protein. Capsid protein is organized such that the protein domain containing tyrosine 180 is exposed on the surface of the capsid. Gentle RNase treatment of crosslinked capsids releases 2 species of protein-containing subunits, having diameters of 9 and 11 nm. These structures represent pentamers and hexamers respectively. The data collectively shows the nucleocapsid to be a t=4 icosahedron in which both protein and RNA are exposed on the surface.

MATURATION OF THE JE E AND NS1 GLYCOPROTEINS. Fan Wufang and Peter W. Mason, YARU, Dept. of Epidem. and Public Health, Yale U. Sch. Med., New Haven, CT.

The maturation of the Japanese encephalitis virus (JE) structural (E) and nonstructural (NS1) glycoproteins in vertebrate and invertebrate cell lines was investigated in order to help define the role of the NS1 protein. E and NS1 were released slowly (half-time > 6 hr) from JE-infected monkey cells (Vero cells). Mosquito cell lines released E at a similar rate, whereas NS1 was retained in an undegraded form in the mosquito cells. The proteolytic processing of these proteins appeared identical in both cell types, but some differences in N-linked glycosylation were observed. E and NS1 found within vertebrate and invertebrate cells contained high-mannose glycans for more than 8 hr after synthesis. Additional sugar residues were added to the single E protein glycan prior to release from Vero cells, whereas sugar residues were trimmed from the E protein glycan prior to release from mosquito cells. The forms of NS1 found in the culture fluid of infected Vero cells contained one complex and one high-mannose glycan. These data indicate that E and NS1 accumulate within an early secretory compartment of the infected cells and then rapidly proceed through the medial- and trans-Golgi compartment prior to release into the extracellular fluid. Transient expression experiments showed that each of these proteins was faithfully processed when expressed in isolation, suggesting that these proteins contain signals that direct them to specific compartments in the infected cell.

ANALYSIS OF THE NONSTRUCTURAL PROTEINS OF THE FLAVIVIRUS WEST NILE VIRUS (WNV)
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The primary structure of the nonstructural proteins NS1, NS2a, NS2b, NS3, NS4b and NS5 of the WNV has been determined. The nonstructural proteins were isolated from nuclear membrane fraction of WNV infected BHK cells. Amino terminal sequence data of these purified proteins were determined. Together with the published amino acid sequence of the nonstructural coding region (Castle et al., 1986, Virology 149, 10-26) we obtained the sequences of the nonstructural proteins NS1 (50 kD), NS2a (19 kD), NS2b (14 kD), NS3 (70 kD), NS4b (27 kD) and NS5 (97 kD). The gene order, the sizes of the virus coded proteins and the processing of the nonstructural proteins appears to be identical between the flaviviruses.

IN VIVO PROCESSING OF DENGUE 2 VIRUS NONSTRUCTURAL PROTEINS

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We have utilized the PATH vector series to produce Trp E-dengue nonstructural protein fusions. The fusions were designed to be specific for a single nonstructural protein. Gel purified antigens were injected into rabbits to elicit production of polyclonal monospecific antibodies. These antibodies were used to study the processing of dengue nonstructural proteins in various cell lines. The intracellular locations of these antigens were determined through the use of indirect immunofluorescence. Differences in both the rate and amount of processing of nonstructural proteins were observed in permissive cell lines. High molecular weight polyproteins are processed into mature lower molecular weight products. It appears that cellular enzymes are intimately involved in the processing of dengue nonstructural proteins and that full-length readthrough polyproteins are the major substrate for the production of mature nonstructural proteins.

THE FUNCTION OF THE SINDBIS VIRUS 6K PROTEIN

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We have used site-directed mutagenesis to study the role of the Sindbis virus 6K protein. The 6K protein is translated as part of the structural polyprotein and was previously detected on membranes of virus infected cells. The protein is strongly hydrophobic and contains 5 cysteines in its sequence of 55 amino acids. We detected high levels of fatty acylation in the 6K protein using radiolabeled palmitic acid. The fatty acyl groups could be removed by neutral hydroxylamine indicating thiol ester linkage to cysteines. Potential sites for fatty acylation in the 6K protein were mutated and two viable mutants were obtained - one with a single cysteine replaced with a serine and one with two adjacent cysteines replaced with a serine-alanine. Both mutants are distinct from wild type in the following properties: (1) they contain less fatty acid in the 6K protein, (2) they contain twice as much 6K protein in the virion, (3) based on plaque assays, both grow poorly in mosquito cells but equal to wild type in avian cells, (4) the rate of release of mutant virus particles from avian cells is 10-20% that of wild type, (5) the specific infectivity (PFU/particle) is 4-8 fold higher for the mutants, (6) the uptake of mutant virus is faster. These data suggest that the 6K protein has a role in virus assembly and also in virus structure.

THE SPECIFICITY OF ASSEMBLY OF THE NUCLEOCAPSID OF SINDBIS VIRUS

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We developed an assay that permits us to identify the sequences in the Sindbis virus RNA which are required for the binding of the viral capsid protein during the first steps of encapsidation. Purified capsid protein was immobilized on nitrocellulose filters and probed with various radiolabeled RNA's transcribed in vitro from different cDNA clones. Using this method we were able to identify a region close to the 5'-end of the Sindbis RNA genome which seems to contain RNA sequences that are important for the specific binding of the viral RNA to the capsid protein.

In addition we were able to demonstrate in vitro reassembly experiments that homologous genomic RNA is preferentially encapsidated in the presence of nonhomologous competitor RNA's to form nucleocapsid like particles.

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RNA SYNTHESIS OF JAPANESE ENCEPHALITIS VIRUS AND EXPRESSION OF NS5 IN *E. COLI*.

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Like other flaviviruses, Japanese encephalitis virus (JEV) genomic RNA has no poly(A) at 3' end and a possibility to be synthesized in a different manner. Synthesis of viral specific RNA in JEV-infected cells was examined by the hybridization using RNA probes. Minus probe could detect negative stranded JEV-specific 42S RNA in the cells as early as 6hr post infection. On the other hand, the amounts of positive stranded 42S RNA increased according to time course of viral reproduction. The positive stranded RNA was found in much greater abundance in the crude membrane fractions. From the data, localization of negative or positive stranded vRNA in the cells was indicated. It was also found that the membrane fraction contained large amounts of non-structural proteins, NS5 and NS3. These results were consistent with the fact that the membrane fraction had high activity of *in vitro* RNA synthesis.

To elucidate the function of NS5, expression of NS5 in *E. coli* was carried out. One of proteins expressed in *E. coli* (100K) showed the same mobility in SDS-polyacrylamide gel electrophoresis as a native NS5 in JEV-infected cells. Biological activity of 100K protein is being investigated.

STUDIES ON THE RHINOVIRUS 14 3C PROTEINASE

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The 3C proteins of several picornaviruses have been demonstrated to be cysteine-type proteinases, involved in processing viral polyproteins. Nucleotide sequencing data indicated that the rhinovirus genome coded for a homologous 3C protein. The HRV14 3C protein, cloned and expressed in *E. coli*, was purified to homogeneity and assayed against various cloned viral proteins. It possessed 'in trans' proteolytic activity, cleaving a CAT-VPg/3C protein, apparently at the authentic VPg/3C junction. Further analysis demonstrated that the 3C proteinase was released from a precursor polyprotein. The HRV14 3C protein was assayed against peptides corresponding to predicted cleavage sites within the polyprotein. Peptides representing the 1B/1C, 2A/2B, 2C/3A, 3A/3B, 3B/3C and 3C/3D sites, with proteolysis predicted to occur at a Gln-Gly junction, were all processed by the 3C protein. Cleavage was specific, occurring at the Gln-Gly bond within the peptide. Peptides corresponding to the predicted 2B/2C and 1C/1D cleavage sites, where the processing was presumed to occur at a Gln-Ala or Gly-Gly bond respectively, were not cleaved by preparations of the 3C protein. The ability of the 3C proteinase to perform the polyprotein cleavages, as well as the cleavages of the synthetic peptides, was inhibited if a Cys 146→Ser mutation was introduced. This data coupled with information from studies with known protease inhibitors, using the synthetic peptides as substrates, lead to the conclusion that the HRV14 3C protein is a cysteine proteinase. The 3C proteinase probably plays an important role in replication of the virus and thus represents a potential target for antiviral chemotherapy.

PROTEOLYTIC PROCESSING OF THE HEPATITIS A VIRUS P3 POLYPROTEIN.

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A putative picornavirus protease 3Cpro was predicted by computation of molecular data of the hepatitis A virus (HAV) genome region P3. The protease was expressed by various means: i) cloning of P3 cDNA into a transcription vector and subsequent *in vitro* translation of synthetic transcripts in rabbit reticulocyte lysates; ii) *in vitro* translation of HAV RNA purified from stool specimens; iii) expression of P3 cDNA in a vaccinia/T7 hybrid system; and iv) infection of cells in culture with HAV. Identification of P3 peptides was achieved with antibodies specific for VPg and 3Cpro respectively. Peptide precursor/product relationships were established through pulse-labeling experiments. From the results we conclude, that HAV RNA codes for a protease 3Cpro, which is expressed in all systems investigated. Proteolytic breakdown of P3 polypeptide precursors in the *in vitro* systems proceeds through intermediates that are expected from the putative P3 structure. However, kinetics of synthetic, and more important degradative events differed significantly among the various systems. Moreover, although mature 3Cpro was generated in HAV infected cells, a 3Cpro related peptide (52K) was detected that does not correlate with the putative P3 structure, and might therefore be implicated in inefficient replication of HAV in cell culture.

EXPRESSION OF PROTEASE 3CD OF POLIOVIRUS TYPE 1 IN *SACCHAROMYCES CEREVISIAE*

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Subunit vaccines are a promising alternative to attenuated or inactivated viral vaccines. Developments in molecular biology and in particular in recombinant DNA techniques have opened new ways for the production of these viral vaccines eliminating the need for production of large quantities of the viral agent.

In view of our interest in producing a subunit vaccine against poliovirus by recombinant DNA techniques, use has been made of *S. cerevisiae* for the synthesis of capsid proteins VP2 and VP1 (1,2).

As one of the criteria for authenticity of VP1 thus obtained, we consider the capability of 3CD to process VP1 into VP0, VP3 and VP1 (3). To this end the DNA fragment encoding 3CD was cloned from pLOP315 (3) and placed in a yeast/*E. coli* shuttle vector under regulation of either the *Gal1* or the *P_{ADK}* promoter.

Preliminary results indicate that constitutive expression of 3CD is lethal to the yeast cells and that induced expression leads to small amounts of an active enzyme.

- (1) Verbakel et al. (1987). Gene 61, 207-215.
- (2) Verbakel et al. (1988). Life Science Advances: Molecular genetics 1.
- (3) Jore et al. (1988). J. gen. Virol. 69, 1627-1636.

IN VITRO CHARACTERIZATION OF RHINOVIRUS (SEROTYPE 2) PROTEINASE 2A UTILIZING A SPECIFIC CLEAVAGE ACTIVITY ON SYNTHETIC PEPTIDE SUBSTRATES

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Proteolytic processing of the polyprotein of human rhinoviruses is carried out by the products of the viral genes 2A and 3C. Protease 2A is responsible for initial cleavage of the polyprotein and possibly occurring co-translationally at the VP1 and 2A regions.

We present an *in vitro* "trans assay" based on the cleavage of an oligopeptide substrate thus providing a convenient test-system in which the conditions could be more exactly controlled.

On expression of the VP1-2A region of HRV2 in bacteria, protein 2A was capable of acting on its own N-terminus: extracts derived of expression systems for mature protease 2A (pE2A) specifically cleaved a 16 amino acid oligopeptide (Ac-TRPITTAGPDSDIYVH) corresponding to the sequence at the *in vivo* cleavage site (Ala/Gly). Constructions which did not cleave the VP1-2A substrate in the bacterial system (e.g. point mutations in the putative active site of 2A) were also inactive with the peptide, indicating that this part of 2A was indeed responsible for proteolysis. Surprisingly no cleavage of peptides could be found with extracts of an original construction (pE18521) formerly used to demonstrate the proteolytic activity of 2A in a bacterial system. This provides evidence that the original cleavage in the bacterial system was an intramolecular event. The inability of the 2A part of pE18521 to act *in trans* is probably due to the presence of 2B and vector derived sequences at the carboxy terminus which reduces the solubility of 2A and thus prevents intermolecular activity. The two expression systems (pE2A and pE18521) are thus complementary and allow the effects of mutations to be examined both on cleavage *in cis* and *in trans*. Furthermore, the peptide assay was used to study the efficiency of *in trans* cleavage using peptides with different lengths or amino acid compositions and to examine the influence of various conditions (e.g. salt concentration, detergents, temperature, pH etc.) on the *trans* activity of 2A.

PROCESSING OF THE STRUCTURAL PROTEINS OF YELLOW FEVER VIRUS.

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The genome of the yellow fever flavivirus (17D-204 vaccine strain) is composed of a single large open reading frame of 10233 nucleotides coding for the precursor to the structural and nonstructural proteins. To analyze the processing of the polyprotein which generates the structural proteins, the corresponding cDNA was inserted downstream of the T7 bacteriophage promoter. RNA transcripts synthesized *in vitro* were translated in reticulocyte lysate. In the absence of membranes, no processing occurred. When microsomal membranes were added, proteins prM and E were translocated. The signals essential for translocation were localized.

These results were confirmed in Spodoptera cells infected with recombinant baculoviruses. The processing of the nonstructural protein NS1 is also being analyzed in this eukaryotic expression system by immunoblot, immunoprecipitation and pulse chase experiments. When proteins E and NS1 are expressed in tandem, both proteins are translocated but the precursor is incompletely processed.

IDENTIFICATION AND FUNCTION OF RUBELLA VIRUS E2 SIGNAL SEQUENCE IN THE TRANSLATION AND CLEAVAGE OF THE POLYPROTEIN. Christian Oster-Blom*, Donald L. Jarvis, and Max D. Summers. Texas A&M University,

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The structural proteins of rubella virus (RV) are translated as a polyprotein (p110) that is processed to produce the virion components, C, E2, and E1. The precise processing mechanism has not been elucidated; however, it must include at least two proteolytic cleavages, to release the individual virion components from the polyprotein, and it must provide for their dichotomous intracellular distribution. The capsid protein, C, remains in the cytoplasm, where it participates in nucleocapsid formation, while the envelope glycoproteins, E2 and E1, enter the secretory pathway, are N-glycosylated, cleaved, and transported to the plasma membrane. Sequence analysis of the 24S mRNA encoding the polyprotein precursor suggests that both E2 and E1 are preceded by signal peptides for translocation across the membrane of the rough endoplasmic reticulum. A recent study has provided direct evidence that the putative signal peptide preceding E1 can, in fact, mediate translocation of E1 (Hobman et al., J. Virol. 62:4259-4264, 1989). In this study, we used the baculovirus expression vector to further examine the processing of the RV glycoproteins. A mutant polyprotein lacking the putative E2 signal peptide, but containing the E1 signal peptide, was not cleaved or glycosylated; instead, it probably was degraded in the cytoplasm. This suggested that the putative signal peptide preceding E2 is functional, and is required for efficient processing of the polyprotein. This conclusion was confirmed and extended by *in vitro* translocation experiments, using a mammalian cell-free system. Again, the results showed that the E2 signal peptide is required for translocation of the E2-E1 polyprotein. The E2 signal peptide appears to be autonomous, as it mediated translocation of E2 in absence of the E1 signal peptide. These experiments revealed that cleavage of the E2/E1 polyprotein requires: (1) the signal peptide preceding E2, (2) microsomal membranes, and (3) sequences beyond the proximal one-half of the E1 signal peptide. This suggests that the proteolytic cleavage that separates E2 and E1 is performed by the cellular enzyme, signal peptidase.

INDIVIDUAL AND COORDINATE EXPRESSION OF RUBELLA VIRUS STRUCTURAL PROTEINS IN COS CELLS.

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Rubella virus (RV) virions contain three structural proteins C, E2 and E1 which are derived by posttranslational processing of a polyprotein precursor. C or capsid protein is a hydrophilic protein which binds to genomic RNA to form nucleocapsids, and E1 and E2 are membrane glycoproteins forming the spike complexes on the virion exterior. In order to study the processing of RV structural proteins and its relation to infectivity and viral assembly, we have constructed RV cDNAs which when transfected into COS cells, allow individual and coordinate expression of the structural antigens. Using oligonucleotide-directed mutagenesis we have located sequences which affect targeting and transport of the RV proteins. We also are using this technique to map the occupied N-linked glycosylation sites in E1 and E2. Immunofluorescence studies in transfected COS cells demonstrated E2 is transported to the plasma membrane in the absence of E1. Translocation of E2 into the endoplasmic reticulum, passage through the Golgi apparatus and to the cell surface appears to be similar to that of Semliki Forest virus p62/E2 glycoprotein in that E1 is not required for these events. Although E1 is not required for the transport of E2 through the exocytic pathway, we have found that the rate of transport is slower in the absence of E1.

BIOSYNTHESIS AND FUNCTION OF CORONAVIRUS SPIKE PROTEIN
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In coronavirus infected cells the spike protein (S) is synthesized and glycosylated on RER membranes. In transitional elements between ER and Golgi membranes the S protein is incorporated into budding virions. Virions are transported through the Golgi apparatus and secreted. During intracellular transport the oligosaccharide side chains are processed which makes the S protein maximally resistant to digestion by endoglycosidase H (endo H). A fraction of the S protein is not incorporated in virions and is probably transported to the cell surface. The biosynthesis and function of the S protein was studied in coronavirus infected cells and in cells infected with recombinant vaccinia viruses expressing the S genes of IBV, MHV and FIPV. By biochemical analysis of intracellular transport using pulse chase labeling of S protein and endo H digestion it was found that the recombinant expression products are transported more slowly to the trans Golgi cisterna than their counterparts in coronavirus infected cells. Syncytia formation was observed in cells expressing recombinant S proteins. Cell fusion was restricted to feline and murine cells for the FIPV and MHV S proteins, respectively.

We propose that the difference in transport rate is due to the incorporation in virus particles. The slow transport of S protein may be interpreted as transient accumulation at or near the site of budding to allow efficient incorporation into budding virions or to localize the budding event itself. The cell fusion data suggest that a specific interaction with a putative cell receptor is necessary to trigger cell-cell fusion.

SPECIFICITY OF ENZYME-SUBSTRATE INTERACTIONS IN FOOT-AND-MOUTH DISEASE VIRUS POLYPROTEIN PROCESSING
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Transcripts derived from a series of FMDV cDNA constructs were translated in a rabbit reticulocyte lysate system. Processing by the L proteinase at the L/1A cleavage site occurred when most of the P1-2A protein was absent. Substitution of sequences upstream of the 2C/3A cleavage site showed that the 3C proteinase was also able to cleave at an entirely novel cleavage site, apparently at K-I amino acid pairs. Cleavage at the 2A/2B site was not only independent of L and 3C proteinases, but occurred when 2A and as few as four 2B N-terminal amino acids were present. Removal of the C-terminal regions of P1-2A and 28C precursors impaired their ability to act as substrates for 3C proteinase activity. Thus, primary processing activities were resistant to changes adjacent to, or at, the site of cleavage whereas secondary processing in trans was sensitive to changes at remote sites.

MULTIVALENT INTERACTION BINDS SFV SPIKES TO THE NUCLEOCAPSID

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The spikes of SFV are composed of two polypeptide chains (E1 and E2) which span the lipid envelope, and a third peripheral polypeptide (E3). Only the E2 polypeptide possesses a cytoplasmic tail. A recent approach using idiotypic network antibodies shows that this cytoplasmic tail and the nucleocapsid have complementary surfaces (Vaux et al., Nature 336, 36-41, 1988), suggesting an interaction between these two entities. We have analysed this postulated interaction by direct biochemical assays using a synthetic peptide corresponding to the amino acid sequence of the E2 tail. This peptide formed oligomers in aqueous solution. Western blotting of the different peptide forms with ³⁵S-labeled nucleocapsid showed that mainly the oligomers bound radioactivity. Because during budding obviously multipoint interactions mediate binding, we attached the peptide to a solid matrix by bridging it to Pansorbin via antibodies recognizing the C-terminal segment of the peptide. This matrix bound the nucleocapsid efficiently and tightly. Excess of soluble peptide partially displaced the binding. Furthermore, SFV glycoproteins bridged to Pansorbin via a monoclonal antibody against the E2 luminal portion bound the viral nucleocapsid efficiently in a solution containing Triton X-100 where soluble spikes did not bind. These results provide biochemical proof for an interaction. We suggest that multipoint attachment is needed to obtain stable spike's tail-nucleocapsid binding.

AUTO-PROTEOLYTIC ACTIVITY OF RECOMBINANT 3C PROTEASE OF HEPATITIS A VIRUS (HAV)

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Picornaviral RNAs encode a single long open reading frame representing a polypeptide of approximately 200 kd. Translation of virion RNA produces precursor proteins which are subsequently cleaved in a series of processing steps to yield the mature viral structural and functional proteins. Comparison of the deduced amino acid sequence of HAV with other picornavirus indicates that 3C of HAV is a cysteine protease. In order to study its substrate specificity the genomic region of 3C was expressed as protein fused to β-galactosidase in E. coli using the pUC and pUR vectors. Extracts of transformed bacteria were tested for 3C antigenicity by immunoblot. Next to the expected fusion protein of about 150 kd proteins of 28, 26 and 17 kd were detected by an antipeptide serum. The N-terminal amino acid sequence of the 28 and 17 kd protein were determined. The data suggest that transformed bacteria produce an auto-proteolytically active protease and a polypeptide produced by internal initiation of translation. The recombinant protease (28 kd) induced antisera which were not able to detect 3C in infected cells but reacted with proteins translated from synthetic RNA. The recombinant protein was insoluble and no transactivity could be measured when recombinant proteins or translation products were used as substrates.

EXPRESSION OF THE GLYCOPROTEIN E OF THE TICK-BORNE ENCEPHALITIS-VIRUS IN E. COLI
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The gene of the glycoprotein E of the tick-borne encephalitis virus (TBE) was expressed in *E. coli* using the plasmid expression system PEX-34. In this system the protein is fused to the MS2 polymerase.
20 monoclonal antibodies (mAbs) raised against the virus E protein were used to analyse the antigenic structure of the fusion protein and to determine which epitopes are expressed. It could be shown that all mAbs, except two of the domain A, reacted in the same way with the fusion protein and the virus protein E.
In order to localize more precisely those epitopes, defined by non-neutralizing mAbs, fragments of the E-gene were expressed and the resulting proteins were analyzed with the mAbs. It could be shown that one epitope (II) is located within the 34 amino-terminal amino acids of the E protein.

"Detection of Virus-specific IgM Antibodies in Patients with an acute Coxsackievirus-B-Infection by μ -Western Blot Technique"
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Current laboratory diagnosis of a CBV (Coxsackie-B-Virus) infection is mainly based on virus isolation, supported by the detection of rising or high virus-specific neutralizing antibody titres. Since such high titres have also been found in apparently healthy people - probably arising by subclinical infection and persisting for a year or more - CBV-IgM detection seems to be a more reliable criteria for serological diagnosis. Since IgM antibodies capture assays have also limitations, we tried to develop a type-specific technique, an improved micro-western blot (μ -WB) - using a diffusion-blot and subsequent immunodetection with biotin-avidin amplified reactions - which enables a rapid and reliable identification of CBV-IgM antibodies. IgM responses found by this test were mostly type-pre-dominant and group-reactive to CBV1, CBV2 and CBV4.

DETECTION AND DIFFERENTIATION OF TICK-BORNE ENCEPHALITIS VIRUS STRAINS BY NUCLEIC ACID SPOT HYBRIDIZATION.
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Nucleic acid spot hybridization with cloned tick-borne encephalitis virus (TBEV, Far Eastern strain Sofjin) cDNA was used for detection of the viral RNA. Detection limit of the test with 32 P-labelled TBEV cDNA as a probe was 10 pg Sofjin RNA. The assay detected different freshly isolated as well as prototype virus strains, the detection limit being 4.3-7.5 lg LD₅₀ units of infectious virus (mice intracerebral infection). The probe cross-reacted with viruses of TBE subgroup and didn't react with non-related flaviviruses of Japanese encephalitis and Dengue subgroups. The viruses of TBE subgroup were differentiated from virus Sofjin by the melting temperature of RNA-DNA hybrids.

Field collected ticks were tested for presence of TBEV RNA. Results of hybridization were in a good agreement with ELISA and virus isolation data.

Synthetic deoxyoligonucleotides complementary to TBEV (Sofjin) genome RNA were used to differentiate TBEV strains and related viruses of TBE sub-group isolated from different geographical areas. These probes revealed genetic heterogeneity of TBEV strains. Number of oligonucleotides hybridizing with a particular virus was assumed as a quantitative measure of similarity to virus Sofjin. A pattern of hybridization of TBEV strains with a panel of oligonucleotide probes correlated significantly with the source of virus strain (strains isolated from patients versus ones isolated from ticks), and correlated only to a small extent with the geographical distribution.

CORRELATION BETWEEN THE LOCALISATION OF VIRAL ANTIGENS AND FLAVIVIRUS-INDUCED STRUCTURES
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Studies on several flavivirus infections in Vero or C6/36 cells revealed a common viral induced structure. This structure was coined as the vesicle. The vesicles are smooth membrane structures of 60-80 nm in diameter. Consistently enclosed within these vesicles was a 'thread-like' structure. These 'threads' have been postulated to be progeny viral RNA. In Kunjin virus infection it was observed that these vesicles appeared at about 10 hr p.i. Their numbers increased exponentially until 24 hr p.i. After 28 hr p.i., these vesicles were without their 'thread' enclosures. This observation was also seen with West Nile, Dengue-2, Japanese and Murray Valley encephalitis viruses although at different times after infection. The cell microtubules were also rearranged in Kunjin and West Nile virus infections. Using immunofluorescence studies the NS3 protein was found to be affiliated with the microtubules. Immunogold technique revealed the NS3 and NS1 proteins to be diffused throughout the cytoplasm but the E protein was present in sparse quantity in the cytoplasm. The NS3 protein was also seen located evenly along the plasma membrane but only clumped deposits were seen for NS1 and E proteins. However none of these proteins were associated with the virus-induced vesicles and their 'thread' enclosures.

ASPARAGINE-LINKED OLIGOSACCHARIDES OF SEMLIKI FOREST VIRUS GROWN IN MOSQUITO CELLS TREATED WITH INHIBITORS OF N-LINKED OLIGOSACCHARIDE TRIMMING

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An increasing interest in glycoproteins and their carbohydrate moieties has led to the development of numerous techniques for the preparation of glycopeptides and oligosaccharides. Semliki Forest virus (SFV) is a good model for such analysis. Previously, we have shown that in cells treated with trimming inhibitors, Endo-H sensitive glycans were formed, and the produced virions were biologically active. In the present study we have analysed the carbohydrate side chains of SFV-envelope glycoproteins, after treatment of *Aedes albopictus* cells with 1-deoxynojirimycin (dNM), deoxymannojirimycin (dMM) or swainsonine (Sw). In vertebrate cells these drugs inhibit the action of glucosidases I, II and mannosidases I and II respectively.

Our results show inhibitor dependent glycosylation of the produced virions. When cells were treated with dNM, the oligosaccharides Glc₃Man₅₋₆GlcNAc were detected; and with Sw, Man₇₋₈GlcNAc were formed; finally, structures such as Man₆₋₇GlcNAc could be found when cells were treated with dMM.

TRANSMISSION OF HEPATITIS E VIRUS TO OWL MONKEYS (*AOTUS TRIVIGATUS*). J Tighehurst*, L Rhodes*, K Krawczynski*, L Asher*, W Engler*, J Caudill*, M Sjogren*, C Hoke*, J LeDuc*, D Bradley*, L Binn*, Walter Reed Army Inst Res, *US Army Res Inst Infect Dis, *Ctrs Dis Control, *Armed Forces Inst Path, USA

Hepatitis E virus (HEV), a proposed designation for the agent of enterically-transmitted non-A, non-B hepatitis, is a positive-strand RNA virus that has caused disease in both hemispheres and in populations likely to have been infected with many enteric viruses during childhood. It has been difficult to establish animal models of HEV infection; among several susceptible species of primates, cynomolgus monkeys have been the most useful. Efforts to understand the virus and its epidemiology have been hampered by limited amounts of HEV and by low concentrations of anti-HEV in most convalescent-phase sera. We inoculated 6 owl monkeys with feces from Mexico known to contain infectious HEV. All seroconverted and had high levels of anti-HEV (detected by IEM) 6 months after inoculation. Three had biochemical and histopathologic evidence of hepatitis, but HEV was not detected in their feces by IEM. HEV antigen was detected by immunofluorescence analysis of liver tissue from 2 of 4 biopsied owl monkeys. (All 5 cynomolgus monkeys given the same inoculum developed hepatitis; one, studied in detail, seroconverted, had HEV antigen in liver, and excreted HEV particles into bile and feces.) Although these owl monkeys did not excrete detectable HEV or uniformly develop hepatitis, all were infected and developed sustained high-level antibody responses that may be valuable for understanding immunity to HEV and for developing rapid immunoassays.

SIMULTANEOUS QUANTITATIVE DETECTION OF MULTIPLE PLANT VIRUSES BY TIME-RESOLVED FLUOROIMMUNOASSAY

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Simultaneous detection of two or more antigens in one sample is of considerable interest in many research areas and in routine diagnostic work. Hosts are often infected by several viruses at the same time and simultaneous detection of two or more viruses is advantageous. Potato plants have often single or multiple infection with potato virus X (PVX), M(PVM), S(PVS), A(PVA) and potato leafroll virus (PLRV). Thus, large scale, routine virus detection in potato plants requires at least six independent DAS ELISA assays for a single plant specimen. Sensitive time-resolved fluoroimmunoassay (TR-FIA) with lanthanide-labelled antibodies opens up the possibility for multiple labelling, since fluorescent lanthanides Eu³⁺, Tb³⁺, Sm³⁺ and Dy³⁺ have narrow banded emission lines clearly distinguishable from each other. For simultaneous quantitative detection of two antigens in one sample, monoclonal antibody (MAb) to PVM was labelled with a lanthanide Eu³⁺ and MAb to PVX with another lanthanide Sm³⁺. A mixture of these MAbs was used for coating and the labelled MAbs were used as a conjugate. After performing the immunoreactions, the fluorescence of the dissociated lanthanides was measured at different wavelengths in a time-resolved fluorometer to quantify PVM and PVX amount in a sample. Double-label TR-FIA detected 1ng/ml of each virus in a single assay. Triple-label TR-FIA, where Eu³⁺, Tb³⁺ and Sm³⁺-labelled MAbs were used, detected 10ng/ml of each potato virus simultaneously. Thus, the quantitative double- and triple-label TR-FIA is at least as sensitive as ELISA.

NUCLEAR ACCUMULATION OF DELIVERED SEMLIKI FOREST VIRUS CAPSID PROTEIN.

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As shown elsewhere, capsid (C) protein of Semliki Forest virus transferred into various target cells by electroporation, liposome, and red-cell-ghost mediated delivery affects protein synthesis in a pleiotropic fashion (Elgizoli et al., J. Virol., in press). Small amounts (10³ to 10⁴ copies per cell) induce the synthesis of specific M₁ classes of cellular proteins, whereas high amounts (10⁵ to 10⁶ copies per cell) act as a general synthesis inhibitor. Here we show that similar to induction, repression of protein synthesis lasted only for about two hours after delivery of C-protein. The repression was followed thereafter by an unexpected, short lived induction of protein synthesis. Evidence is presented showing that delivery of C-protein into the cytosolic compartment resulted in its rapid confinement to the nucleus at the expense of C-protein present in the cytoplasm. In addition, morphological studies as well as subnuclear fractionation revealed that the majority of the delivered C-protein molecules had a high affinity for nucleoli.

TRYPSIN SENSITIVITY OF SEVERAL HUMAN RHINO-VIRUS SEROTYPES IN THEIR LOW pH-INDUCED CONFORMATION

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Human rhinoviruses were examined for sensitivity to trypsin at physiological pH and after exposure to low pH. HRV1A, HRV2 and HRV14 were found to be resistant whereas in serotypes HRV49 and HRV89 degradation of VP2 was observed. However, exposure to low pH led to rapid cleavage by trypsin of VP1 in HRV1A, HRV2 and HRV49 at defined sites followed by degradation of VP2. The cleavage site in VP1 was determined for HRV2 and was shown to occur between Arg260 and Thr261, close to the C-terminus. As the cleavage site is most probably buried inside the capsid, structural rearrangements of the viral capsid are thus necessary to account for the cleavage observed after low pH treatment.

TYPING OF HUMAN RHINOVIRUSES BASED ON SEQUENCE VARIATIONS IN THE 5' NON CODING REGION

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Unambiguous assignment of restriction enzyme patterns to six individual serotypes (two sets of closely related ones, HRV1A and HRV1B and HRV2 and HRV49 together with the much less closely related HRV14 and HRV89) of human rhinovirus was accomplished after amplification of a 380 bp DNA fragment derived from the 5' non-coding region using the PCR technique. This was possible even though the closely related serotypes 1A and 1B and serotypes 2 and 49 differed only in 10 and 17 nucleotides respectively in this region. This method utilizes the conserved and variable components of this part of the genome and provides the basis for a simple and rapid typing of human rhinoviruses.

CHARACTERIZATION OF RUBELLA VIRUS GLYCOPROTEIN E2 OLIGOSACCHARIDES.

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Rubella virus glycoprotein E2 is a constituent of the viral envelope. It has a molecular weight of 42,000-47,000 D and is highly glycosylated. The fully processed E2 found in mature virions is a very heterogeneous species, which is believed to be due to differences in glycosylation. The difference in MW between unglycosylated and fully processed E2 is more than 12,000 D, suggesting that at least one of the three available glycosylation sites becomes glycosylated with an unusually large oligosaccharide. The high degree of heterogeneity of mature E2 also indicates that the carbohydrate moiety is subject to extensive processing.

In order to determine the structure of the carbohydrate moiety of E2, the structural proteins of the rubella virus strain M33 were purified using polyacrylamide gel electrophoresis and subsequent electroelution of protein bands. Purified E2, radiolabelled with ³H-GlcNAc, was digested with pronase and the resulting glycopeptides were analyzed using HPLC, column chromatography and Sepharose- or Agarose-bound lectins. Glycopeptides were also digested with various glycosidases and the change in size was monitored by gel filtration.

PERSISTENT INFECTION OF K562 CELLS BY ENCEPHALOMYOCARDITIS VIRUS

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Based on the paucity of published reports, it appears that establishment of persistent infections by encephalomyocarditis (EMC) virus does not occur readily. It was surprising, therefore, to find that persistently infected cultures were established with ease when EMC virus infected human leukaemic K562 cells. In contrast to the usual typical lytic infection by EMC virus where trypan blue staining of cells reaches close to 100% by about 15 hr post-infection, K562 cell cultures required 3 to 4 days following infection to reach a maximum of about 80% cells stained. The proportion of K562 cells taking up stain gradually decreased to about 10% of those present by about 13 days post-infection; during this time, virus yield per day measured by either plaque or haemagglutination titration fell about ten fold. In some cultures, this decrease in per cent staining was followed by later waves of increased staining accompanied by increased virus production. Virus-producing cultures have been maintained for over 3 months. Evolution of both virus and cells accompanied the establishment of persistence in that plaque size changed from about 7 mm in diameter for the original virus to less than 1.5 mm by day 20 post-infection and the majority of cells cloned from persistently infected cultures were resistant to superinfection with original virus. Resistance was due, at least in part, to reduced virus attachment in that binding of ³H-virus to cloned resistant cells was about 1.7% of that to uninfected cells. The possible presence of viral products in the resistant cells and defective interfering particles in the virus population are presently under investigation as part of this continuing study. Work supported by the Canadian MRC and the Canadian Diabetes Association.

MECHANISM OF ASTROVIRUS ENTRY INTO 293 CELL LINE
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In order to investigate the cell entry pathway of serotype 1 human astrovirus, different lysosomotropic agents (ammonium chloride, methylamine and dansylcadaverine) and a ionophore (monensin) have been evaluated in their ability to interfere with the infection of a human embryo kidney cell line (293). In fact, these compounds are known to raise endosomal and lysosomal pH by different mechanisms. Virus attachment to cell monolayers was allowed to occur at 0°C; then viral penetration was induced by temperature shift at 37°C. Rate of infection was monitored by indirect immunofluorescence after 24 hours. The findings of our experiments showed that all drugs tested were not virucidal and did not interfere with the virus attachment step. On the other hand when the same compounds were added following viral binding, antigen synthesis was significantly inhibited. All drugs acted on the early stages of infection and ammonium chloride was the most effective. Results obtained suggest that human astrovirus entry follows an intravesicular route requiring a pH dependent process for viral genome release in the cytosol. These data have been supported by electron microscopy observations on infected cells in which viral particles have been detected within coated vesicles.

CREATION OF AN ANTIGENIC SITE IN POLIOVIRUS TYPE 1 BY ASSEMBLY OF 14 S SUBUNITS
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14 S subunits are pentamers of the structural unit comprising one molecule of each of the capsid polypeptides VP0, VP1 and VP3. These 14 S subunits are assembled into virions. On poliovirions, four different antigenic sites have been recognized. Site 1, which is immunodominant in poliovirus type 3, but of lesser importance in type 1, is continuous, whereas the structure of the other antigenic sites appears to be more complex.

Nineteen neutralizing monoclonal antibodies targeted against the four known antigenic sites of poliovirus type 1 were tested for their binding capacity of 14 S subunits. All 13 antibodies targeted against antigenic sites 1, 2 and 3A recognized 14 S subunits, whereas none of the 6 antibodies specific for site 3B did so.

It may be concluded that three out of the four major antigenic sites (1, 2 and 3A) are already present on 14 S subunits. Site 3B, in contrast, is only present on virions and spans the boundary between pentamers in the virion. As this antigenic site is created by assembly of 14 S subunits it might be called a "neotope".

ANTIGENIC PROFILE OF CHIKUNGUNYA VIRUS
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Chikungunya virus (CHIK) is an alphavirus endemic to Africa and Asia, where it causes human disease characterized by fever, arthralgia, and frequent arthritis. To facilitate efforts in the development of vaccines protective against the greatest possible array of medically important alphaviruses, we initiated studies of CHIK immunobiology. Assays with polyclonal sera underscored the natural polymorphism of neutralization epitopes: not only was there poor cross-neutralization with other members of its antigenic complex (e.g., Semliki Forest, Mayaro, and Ross River viruses), there was considerable heterogeneity among various CHIK isolates. However, antibody-mediated complement-dependent cytotoxicity, known in many cases to be a correlate of protection by non-neutralizing antibodies, demonstrated great similarity among CHIK strains and some potential for cross-reactive protection with disparate alphaviruses. Monoclonal antibodies were used to establish that two conserved epitopes were homologous to E1 topologic sites previously shown to be involved in protection against Sindbis virus, while other cell-surface epitopes, not yet characterized in detail, were restricted to CHIK and closely related viruses. Unlike humoral responses, lymphoproliferative assays -- taken as crude measures of T cell responsiveness -- did not reveal cross-reactivity between CHIK and distantly related alphaviruses, Sindbis and Venezuelan equine encephalitis viruses.

MONOCLONAL ANTIBODIES TO DENGUE TYPE 1 NONSTRUCTURAL PROTEIN NS3

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Dengue viruses of which there are 4 serotypes cause dengue fever and the more severe dengue haemorrhagic fever/dengue shock syndrome. In animal protection studies, envelope proteins of dengue 2 virus, contrary to conventional thinking, did not provide significant protection against dengue virus challenge. In contrast, passive protection in mice has been demonstrated with monoclonal antibodies to NS1 of dengue 2 virus and active protection using NS1. The aim of this project was to study the immunological significance of the nonstructural protein NS3 using monoclonal antibodies prepared against dengue 1 virus. The monoclonals produced were selected by ELISA using infected C6/36 cell lysate which has been found to contain nonstructural proteins in western blots when tested against mouse hyperimmune serum. ELISA positive hybrids were cloned by limiting dilution twice and further characterised by western blot. An indirect immunofluorescent assay (IFA) was used to confirm the presence of dengue virus antibodies. Four monoclonal antibodies that were positive in IFA were selected for further studies. These showed no reactivity in complement fixation and haemagglutination inhibition tests. By radioimmuno-precipitation using [³⁵S] methionine labelled C6/36 cell extract, these antibodies precipitated two proteins of molecular weights similar to that of NS3 and NS5. These monoclonals reacted in western blot to a Trp E-NS3 fusion protein expressed from a recombinant plasmid, pATH 10 NS3(Eco-Eco)S [provided by V. Deubel]. This fusion protein contains approximately 40% of the carboxy terminal of the NS3 protein. Antibody studies in patients with the Trp E-NS3 fusion protein as well as passive protection studies with the monoclonals produced will be performed.

NEUTRALIZATION OF POLIOVIRUS

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Among different mechanisms of virus neutralization one seems to be essential which is suggested to stabilize the capsid by cross-linking subunits with bivalent antibodies and thus preventing the virus from being uncoated. This is supported by the observation that after cleavage of the antibodies into Fab fragments, virus infectivity is completely restored, while the Fabs remained on the virus surface. This effect could be simulated by cross-linking poliovirus with a bifunctional reagent by introducing disulfide bridges between subunits, which reduced infectivity by more than 99%. It could be shown that VP1-VP3 were cross-linked. Upon cleavage of the cross-links using mercaptoethanol, infectivity was restored as well. It is therefore suggested that neutralization occurred only if antibodies are bivalently bound. There is evidence that virus which has been neutralized using different methods penetrated into HeLa cells. Virus treated with Glutathione could be reisolated from infected cells and was fully infective. Likewise was chemically cross-linked virus and antibody-treated virus reisolated from cells, while antibodies alone did not enter cells. It is suggested that upon chemical cross-linking of subunits at the virus surface with few bridges a cooperative conformational change of the virus shell is blocked, which, under liberation of the RNA, leads to an infection. A similar mechanism probably occurs with antibody-bound virus.

ANTIGENIC SITE-SPECIFICITY OF ANTIBODY RESPONSES TO TYPE 3 POLIOVIRUS IN MICE

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Groups of Balb/c mice were immunized with intact or trypsin-cleaved type 3 poliovirus/Saukett or with a recombinant poliovirus containing, in the type 1/Mahoney basic structure, the exposed trypsin-sensitive BC loop of VP1 as the only component from the type 3/Leon virus (Ref.1). Similar preparations were used as challenge virus in assays measuring neutralizing antibodies.

Intact type 3/Saukett virus induced antibodies targeted mainly to the BC loop of VP1, which is a central part of the designated antigenic site 1. The level of these antibodies decayed rapidly while the response to other antigenic sites became more prominent during the three weeks' observation period. Mice immunized with the trypsin-cleaved type 3/Saukett virus also developed antibodies to the BC loop of VP1 but the proportion of antibodies targeted to other antigenic sites was greater than in the case of the intact virus-immunized mice. Recombinant virus-induced type 3-specific antibodies were all targeted to the BC loop of VP1 and consequently, could not neutralize trypsin-cleaved type 3 polioviruses.

These results suggest that by using trypsin-cleaved poliovirus as an injectable immunogen it is possible to modify the antigenic site distribution of the induced antibodies towards a pattern similar to that observed in man after poliovirus infection.

References:

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RECEPTORS FOR RUBELLA VIRUS ON VERO CELL MEMBRANES

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Membrane receptors for rubella virus (RV) in Vero cells were studied by means of two different approaches: i) by enzyme treatment of the whole cell membrane and ii) by testing the ability of isolated plasma membrane molecules to compete with cells for virus binding. The susceptibility of enzyme treated cells to RV infection was assessed by indirect immunofluorescence assay. Phospholipases A2 and C digestion greatly reduced the infectivity by the virus, suggesting the involvement of lipid structures as receptor sites for RV. When the major membrane lipids were examined separately for their ability to inhibit viral infectivity, several phospholipids (phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin) and glycolipids (cerebroside sulphate, lactosylceramide, gangliosides) showed a strong neutralizing activity. This effect was dose dependent. The replication of lipid treated rubella virions was studied with both molecular hybridization techniques and indirect immunofluorescence assay. RV specific recombinant plasmids were used as probe for the detection of virus sequences in the various experimental conditions.

EFFECT OF AMMONIUM CHLORIDE AND CHLOROQUINE ON THE GROWTH OF RUBELLA VIRUS IN VERO CELLS

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To study the penetration and uncoating processes of Rubella virus (RV), we analyzed the effect of ammonium chloride and chloroquine, known to increase the pH of intracellular vesicles, on the infectivity of RV in Vero cells, under one-step multiplication conditions. The lysosomotropic weak bases inhibited viral replication monitored by indirect immunofluorescence assay. A total inhibition was achieved when ammonium chloride (20 mM) and chloroquine (0.05 mM) were present during a complete replication cycle (48 hr), after viral adsorption on cell membrane (1 hr, 4 °C). The time course for the inhibition during the first 24 hours was determined by adding/removing the drugs at different times after the virus addition. The presence of the agents in the first 2 hours did not modify viral replication. At subsequent time there was a time-response relationship with 50% inhibition at 6 hours and a 100% inhibition at 12 hours. The time required for viral infectivity inhibition by the lysosomotropic agents corresponded to the latent period of the virus and to the first detection of viral RNA synthesis.

EFFECT OF POLYIONS ON SINDBIS VIRUS-CELL INTERACTION
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The involvement of electrostatic interactions in the binding of Sindbis virus to cell membrane receptors was studied using several polyanions and polycations. The capacity of polyions to interfere with viral attachment was tested on Vero cells, susceptible to infection, and on binding and fusion of goose erythrocytes. The agents were incubated with Vero cells immediately before, during or after exposure of the cells to Sindbis virus. Heparin inhibited plaque formation only when present during virus adsorption. In contrast poly-L-lysine and protamine had inhibitory effects whether they were incubated with cells before or during exposure to virus. None of the polyions was able to inhibit viral attachment to erythrocytes, whereas the hemolytic activity was sensitive to mucin and polygalacturonic acid. All these results suggested a role of membrane proteoglycans as Sindbis virus binding sites; therefore the effect of heparinase and chondroitin ABC lyase digestion on the susceptibility of cells to viral binding has been studied.

FUNCTIONAL ANALYSIS OF A PANEL OF MONOCLONAL ANTIBODIES GENERATED AGAINST THE NON-STRUCTURAL GLYCOPROTEIN, NS1 OF DENGUE TYPE 2 VIRUS (PR159).

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Antibody mediated enhancement of viral replication has been implicated in the pathogenesis of dengue haemorrhagic fever. In an attempt to circumvent the problems associated with eliciting an immune response directed against structural components of the virion, the protective capacity of the non-structural glycoprotein, NS1 has been assessed. Both active and passive protection with homologous but not heterologous dengue virus serotypes have now been demonstrated. While common epitopes within the dengue group have been identified by a number of workers using polyclonal sera, no monoclonal antibodies (mAbs) have been described which would allow a detailed analysis of potential cross-protective epitopes.

In this study, affinity purified dimeric NS1 of dengue 2 (PR159) virus was obtained using high pH elution. MABs obtained from mice immunized with this protein have yielded a number of dimer specific clones as well as a panel of mAbs showing a variety of cross-reaction patterns within the dengue group. Several clones describe both linear and conformational epitopes common to all 4 serotypes of dengue viruses. A number of these clones also cross-react with other flaviviruses, most notably with members of antigenic group III. These mAbs have been studied for their ability to fix complement, lyse virus infected target cells *in vitro* and to confer passive protection in mice against live dengue virus challenge.

MURINE T LYMPHOCYTE RESPONSES TO DENGUE VIRUS AND VIRAL PROTEINS

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We have studied the *in vitro* responses of spleen cells from dengue virus-immune mice as a model of human lymphocyte responses to dengue virus. Mice were immunized with one intraperitoneal dose of live dengue virus. Spleen cells from mice immunized with any of the four dengue serotypes showed significantly higher levels of proliferation when incubated with an antigen of Vero cells infected with the same dengue serotype than when incubated with a preparation from uninfected Vero cells. The proliferating cell population was predominantly Th1.2⁺ L3T4⁺ Lyt2⁻. This proliferative response was predominantly serotype specific, with a lower level of serotype cross-reactivity.

Viral proteins expressed in Sf9 cells with a recombinant baculovirus were used to identify viral proteins responsible for the proliferation. Lymphocytes from dengue 4 virus-immune mice showed high levels of proliferation in response to a preparation expressing dengue 4 virus C, pre-M, E, NS1, and NS2a proteins. A preparation expressing only dengue 4 virus E protein also induced a proliferative response, although to a lesser extent.

T cell clones have been established from dengue 2 virus-immune mice by repeated stimulation with dengue 2 virus antigens. The clones express the Th1.2⁺ L3T4⁺ Lyt2⁻ phenotype. Proliferative responses of the clones are predominantly serotype specific, similar to the responses of immune spleen cells in bulk culture.

IDENTIFICATION AND PRESENTATION OF THE ANTIGENIC SITES OF O₁ KAUFBEUREN FOOT-AND-MOUTH DISEASE VIRUS

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Four antigenic sites have been identified on O₁ Kaufbeuren FMDV by the characterization of monoclonal antibody escape mutants. Two sites involve amino acid residues from VP1, a third site residues from VP2 while the fourth site consists of residues from VP3. Polio/FMDV chimaeric viruses have been constructed, with sequences corresponding to the antigenic regions of FMDV inserted into antigenic site 1 of poliovirus. One of these polio/FMDV chimaeras is neutralised by anti-FMDV mAbs and polyclonal sera. The immunogenicity of these viruses is being investigated.

THE CONSTRUCTION AND CHARACTERIZATION OF POLIOVIRUS: HIV-1 ANTIGEN CHIMAERAS

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Following the successful construction of an intertypic poliovirus chimera by Burke *et al.*, (Nature 332:81-82) we have used antigenic site 1 of the live-attenuated Sabin type 1 vaccine to present epitopes from Human Immunodeficiency Virus (HIV-1). Viable antigen chimaeras have been generated that express known and predicted epitopes from the envelope glycoproteins (gp120, gp41) of the HTLV-IIIB strain of HIV-1. The antigenicity and immunogenicity of the chimaera, containing 18 residues derived from gp42 has been shown to induce group specific neutralizing antibodies in rabbits. We have demonstrated that antigenic site 1 displays considerable flexibility, both in the number and sequence of amino acids that can be accommodated. These studies establish the potential of poliovirus as a vector for the expression of foreign epitopes, which may have implications for future vaccine design.

RECEPTOR GROUPING OF ENTEROVIRUSES

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Cell tropism and host range restriction in picornaviruses is largely determined by cellular receptor specificity. It has been shown that each of the >100 human rhinovirus serotypes bind to human cells via one of only 2 classes of receptor. Similarly, the 3 serotypes of poliovirus bind to a common, unique, receptor. However, very little grouping of the >30 other human enterovirus serotypes according to their receptor specificities has been performed. Human-rodent somatic cell hybrid lines are being used to perform such grouping. The chromosome complements of survivors of virus infection are being studied to establish the chromosomal location of the receptor genes.

PRELIMINARY CHARACTERISATION OF THE RECEPTORS INVOLVED IN KUNJIN VIRUS ENTRY VERO CELLS

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Evidence have shown that entry of Kunjin virus is via receptor mediated endocytosis. Data from interference studies showed the receptors to be unique to Kunjin virus. When West Nile or Sindbis viruses were exposed to Vero cells simultaneously with Kunjin virus the rate of entry of the viruses were similar to that of single infection by each virus. There was also no interference when Sindbis or West Nile virus was coinfecting with Kunjin virus. The intracellular virus yields after the adsorption period was similar to single infection by each of these virus. Further characterisation was made using a range of proteases and glycosidases. The preliminary data showed that the entry of Kunjin virus was inhibited by pretreatment of the cells with these enzymes, indicated the receptor may be a glycoprotein. Initial results show that the activities of alpha-glucosidase and alpha-galactosidase appear to inhibit virus adsorption.

DETECTION OF THE HUMAN RHINOVIRUS MINOR GROUP RECEPTOR ON RENATURING WESTERN BLOTS

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The human rhinovirus minor group receptor was extracted from HeLa cell membranes and partially purified. Receptor activity was detected on Western blots by binding of ³⁵S labelled human rhinovirus serotype 2 (HRV2) to the immobilized protein at a position corresponding to a molecular weight of 120 kD. The properties of the filter-immobilized receptor were assessed and found to be very similar to those of the HeLa cell membrane-associated protein.

MODELING OF THE C1 DOMAIN OF INTRACELLULAR ADHESION MOLECULE 1 (ICAM-1), THE HUMAN RHINOVIRUS MAJOR GROUP RECEPTOR.

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ICAM-1 has recently been shown to be the cellular receptor for the major group of rhinoviruses (Staunton et al. (1989) *Cell*, 56, 849; Greve et al., (1989) *Cell*, 56, 839). The sequence of ICAM-1 suggests it is a member of the immunoglobulin supergene family and is consistent with five immunoglobulin like domains (Staunton et al., (1988) *Cell*, 52, 925). The C1 domain of ICAM-1 is modeled using structural and sequence information to gain insight into the viral-cell surface interaction. The starting point for the ICAM-1 modeling is the known structures of the CL, C1, C2, C3 IgG domains. These domains share a common seven strand beta sandwich secondary structure which allows them to be structurally aligned. The sequence of the C1 domain of ICAM-1 is aligned with the sequences of the immunoglobulin domains with known structures, which allows the formulation of three dimensional model of the ICAM-1 C1 domain. The model permits docking of complementary surfaces and charges between the Human Rhinovirus-14 canyon and the ICAM-1. The ICAM-1 C1 domain model fits well into the rhinovirus canyon where it covers residues which are known to be important in viral attachment to cells, as determined from mutational analysis (Colonna et al., (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 5449).

COMPONENTS OF NEUTRALIZATION: THEIR IMPACT ON VARIATION IN A POLIOVIRUS NEUTRALIZATION ASSAY

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The neutralization assay for measuring antibodies to poliovirus has been used extensively to determine efficacy of vaccination policies worldwide and an individuals resistance to possible infection. Despite nearly four decades as the cornerstone of *in vitro* measurements of host immunity, many components of this assay have not been carefully considered during efforts to interpret results between different tests both within and among different laboratories. We have examined the effect of 1) the possible mechanisms of neutralization, 2) the variation in virus concentration, 3) the variation in end point estimation, and 4) mechanical and logistic factors as they affect variation. We have used this information to implement several practical modifications and refinements to the microneutralization assay and applied these changes to a project involving 24,000 neutralization assays performed over a period of less than one year. Use of these procedures gives an accurate method to correct for daily differences in test sensitivity. This approach to test development has implications for many biological measurements of the virus-host interaction.

RECOGNITION OF FLAVIVIRUSES BY T HELPER CELLS

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Antibody is considered to be the main mechanism of protection from flaviviruses and since generation of this response requires helper T cells we have prepared cloned lines of flavivirus-specific CD4⁺ T cells. These have been used to determine T cell recognition sites on these viruses and also for studying T cell cross-reactivity between members of this family. Our cloned lines of Murray Valley encephalitis-specific murine T cells recognize a linear sequence of the envelope protein and supply help for antibody production following stimulation by other viruses of the West Nile sub-group. However, this site does not strongly stimulate polyclonal virus-immune cultures. These appear to recognize, in addition, nucleocapsid and non-structural proteins some of which also stimulate weakly. It has been necessary to prepare continuous lines of virus-immune T cells to determine the amino acid sequences which are involved.

ANTIGENIC STRUCTURE OF THE COAT PROTEINS OF POTATO VIRUS X (PVX) AND OF POTATO AUCUBA MOSAIC VIRUS (PAMV)

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PVX and PAMV coat proteins were cleaved by cyanogen bromide and staphylococcal protease V 8. Peptides were isolated by means of gel-filtration, ion-exchange chromatography and HPLC. Some of the peptides were purified to homogeneity and their amino acid sequence was established.

With monoclonal antibodies (Mabs) to PVX using indirect ELISA, europium time-resolved fluoroimmunoassay and electro-blot immunoassay we located one or two overlapping antigenic determinants at the N-terminal region of the coat protein. Lysine at position 19 of the virus coat protein is required for the interaction with two of the Mabs. Synthesis of peptides, as a probable epitopes, was carried out by solid-phase method, bases on Fmoc- and Boc-approaches. The synthetic peptides were conjugated to BSA in varying molar ratios by new cross-linker obtained in our laboratories and the immunological reactivity of the conjugates compared.

Mabs to PAMV were raised and used to study the antigenic structure of PAMV coat protein, as well as the immunological cross-reactivity with PVX and its protein.

MOLECULAR GENETICS OF THE MHV RECEPTOR

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The cellular receptor for mouse hepatitis virus (MHV-A59) is a 100-110K glycoprotein found on liver and intestinal plasma membranes. Removal of carbohydrate groups from the protein with endoglycosidase F does not destroy virus binding activity which suggests that the E2 viral envelope glycoprotein recognizes the protein core of the receptor. The receptor has been purified from detergent-solubilized mouse liver membranes by affinity chromatography using monoclonal anti-receptor antibody. The first 15 amino acids from the N-terminus were determined by microsequencing. An antibody was generated in rabbits against a synthetic peptide with this sequence. The anti-peptide antibody recognized the affinity purified receptor from liver and intestinal brush border membranes from susceptible mice. The antibody also recognized an intestinal brush border protein from resistant SJL/J mice. The SJL/J protein was 5-10K shorter than the MHV receptor protein of BALB/c mice, and did not bind either the virus or the virus blocking monoclonal antibody. SJL/J mice may be resistant to MHV due to a mutation in the receptor glycoprotein resulting in a molecule that does not bind MHV.

A 35-mer oligonucleotide deduced from the amino acid sequence of the amino terminus of the receptor was synthesized and used to probe lambda gt11 cDNA libraries made from cells expressing the receptor. Fourteen independent cDNA clones have been isolated. Cloning of the MHV receptor cDNA will allow direct comparison of the receptor protein and the virus non-binding variant of the SJL/J mouse.

INTERTYPIC POLIOVIRUS CHIMAEAS

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The antigenicity and structure of poliovirus have been studied, allowing the location of the three major antigenic sites involved in neutralization on the x-ray crystallographic model of the virus. Based on this information, intertypic poliovirus chimaeras, involving all three neutralizing sites, have been constructed via oligonucleotide-directed mutagenesis of infectious Sabin type 1 and 3 poliovirus cDNAs. Characterization of such antigen chimaeras has been carried out using monoclonal and polyclonal antisera in neutralization and single radial immunodiffusion antigen blocking tests. In addition reactivity, of antisera raised against the chimaeras in mice, rabbits and guinea pigs has been studied. The chimaeras are also being used to examine the immune response of animals and man to different antigenic sites. The information gained from the characterization of intertypic chimaeras leads to a further detailed understanding of poliovirus antigenicity and immunogenicity.

1. Minor et al., 1986. *Journal of General Virology* 67: 1283-1292.
2. Hogle et al., 1985. *Science* 229: 1358-1365.

SYNTHESIS AND IMMUNOREACTIVITY OF DENGUE 2 VIRUS (FLAVIVIRUS) MODIFIED ENVELOPE PROTEIN E USING RECOMBINANT BACULOVIRUSES.

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We examined the expression of the dengue (DEN) 2 virus-specified envelope protein in *Spodoptera frugiperda* SF9 cells by recombinant baculovirus vectors. A cDNA copy of the DEN 2 virus region encoding the virion structural proteins was inserted into the baculovirus DNA using the pACYM1 shuttle vector (gift from Pr D.H.L. Bishop, Oxford). The protein E was produced in a size and antigenicity similar to the corresponding polypeptide from DEN 2 virus-infected *Aedes albopictus* C6/36 cells.

Deletions of respectively 25 and 71 amino acids in the C-terminal end of the protein E produced truncated forms which were secreted in the extracellular medium. Deletions in the C-terminus apparently did not modify the correct presentation of the important epitopes as determined by immunofluorescence and Western blotting using polyclonal and monoclonal antibodies (gift from Drs J.J. Schlesinger and M.K. Gentry). Results of immunization in rabbits and mice with the engineered protein E will be presented.

EPI TOPE ANALYSIS OF ANTIGENIC DOMAINS OF DENGUE 2 VIRUS (FLAVIVIRUS) ENVELOPE PROTEIN E EXPRESSED IN E. COLI.

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Several fragments of the dengue (DEN) 2 virus envelope protein E were expressed in *Escherichia coli* as fusion proteins. The hybrid proteins were synthesized in large amounts by *E. coli* under the control of promoter trpE. The hybrids between trpE and the envelope protein were purified from soluble (amino acids N°59 to 97 in the DEN 2 protein E) or insoluble (amino acids N°59 to 422, 22 to 205, 259 to 422 in the DEN 2 protein E) fractions of transformed *E. coli*.

The immunoreactivity of the hybrids was examined by Western blotting. Two domains contained epitopes reacting with monoclonal antibodies (MAbs) of group, complex and/or type specificity.

Most of the reacting MAbs, and in particular the DEN 2-specific neutralizing MAb 3H5, recognized the domain including amino acids N°259 to 422 in the protein E. The reactivity of all these MAbs was destroyed when this domain was split in two parts expressed independently (amino acids N°259 to 353 and 366 to 422), suggesting the presence of conformational epitopes. The missing peptide (N°354 to 365) contains two proline residues that might also be important for the secondary structure and therefore for the immunoreactivity of the domain.

The immunological response in rabbits and in mice immunized with these fusion proteins will be presented.

DENGUE VIRUS-SPECIFIC HUMAN CYTOTOXIC T LYMPHOCYTES (CTL) Ichiro Kurane¹, Jack F. Bukowski, Ching-juh Lai², Margo Brinton³, Michael Bray², Barry Falgout², Bangti Zhao², and Francis A. Ennis¹. 1. Univ. of Massachusetts Medical Ctr., Worcester, MA, USA. 2. NIAID, NIH, Bethesda, MD, USA. 3. Georgia State Univ., Atlanta, GA, USA.

We have begun to analyze human T cell responses to dengue viruses. We established twelve CTL clones from the lymphocytes of a donor who had been infected with dengue 3 virus. These clones have a CD3⁺ CD4⁺ CD8⁺ phenotype. They lyse dengue antigen-pulsed and dengue virus infected autologous lymphoblastoid line (LBL) in an HLA class II-restricted fashion. One of the clones lyses target cells expressing dengue 4 NS1, 2a, 2b, 3, 4a and 4b, but does not lyse target cells expressing E, Pre M, C, NS1 and 2a or cells expressing NS1 and 2a. This result suggests that this clone recognizes an epitope on a non-structural protein other than NS1, NS2a, and NS5. Purified dengue 3 NS3, but not NS1 or NS5, induces a high level of proliferation. A short-term T cell line stimulated with NS3 has a CD3⁺, CD4⁺, CD8⁺ phenotype and lyses dengue 2 virus-infected LBL. This result suggests that an epitope on NS3 is recognized by CD4⁺ T lymphocytes. We have also detected serotype cross-reactive dengue-specific, HLA class I-restricted, CD4⁺ CD8⁺ CTL in bulk culture. These CTL lyse autologous fibroblasts infected with a vaccinia construct which contains NS1, 2a, 2b, 3, 4a and 4b, and lyse target cells expressing E and those expressing E, Pre M, C, NS1 and 2a to a lower level. Target cells expressing NS1 and 2a are not lysed. These results suggest that dengue-specific, HLA class I-restricted CTL recognize mainly non-structural proteins other than NS1 and 2a, and also recognize E to a lower level. CD8⁺ cross-reactive CTL clones have also been established.

IDENTIFICATION OF CONTINUOUS EPITOPES OF THE ENVELOPE GLYCOPROTEIN OF DENGUE TYPE 2 VIRUS

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Four hundred ninety hexapeptides homologous to the amino acid sequence of the dengue 2 envelope glycoprotein were reacted with antisera from seven patients with primary dengue 2 infections to identify the continuous epitopes recognized by human IgG. There were 124 peptides in twenty five clusters (domains) that bound two or more antisera. Twenty two peptides in seven domains bound all seven convalescent dengue 2 antisera tested and thus appeared to represent immunodominant epitopes. The evidence that these domains represent continuous epitopes of the envelope glycoprotein is: (1) peptides representing each domain bound multiple sera, (2) peptide reactivity was highly ordered along the amino acid sequence and (3) in almost all cases, domains were regions of predicted hydrophilicity. Heterologous flavivirus antisera also exhibited binding to the majority of peptides reactive with anti-dengue 2 sera though four candidate dengue 2 specific epitopes were identified along with an immunodominant epitope common to dengue, Japanese encephalitis and West Nile viruses. Synthetic peptides representing these epitopes may prove to be useful for a variety of purposes.

EFFECTIVE EXPRESSION OF ANTIGENICALLY ACTIVE STRUCTURAL AND NONSTRUCTURAL PROTEINS OF JAPANESE ENCEPHALITIS VIRUS

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Recombinant Baculoviruses and Vaccinia viruses containing the coding sequences of the structural and/or nonstructural proteins of Japanese encephalitis virus were constructed. The antigenic properties of the expressed proteins by the recombinants were evaluated using a panel of monoclonal antibodies against the E protein of JE virus and polyclonal antibodies and the molecular size of the proteins were analysed by Western blotting. All the epitopes detected by a panel of monoclonal antibodies were demonstrated on the E protein expressed by the recombinants which had the coding sequence of preM and NS1 to add to E. The E protein was glycosylated and same in size to the authentic E and transferred to the surface of the recombinant infected cells. However, the E proteins expressed by the recombinants which had no preM sequence or truncated E sequence showed no reactivity with the monoclonal antibodies and were not demonstrated on the cell surface. The NS1, another membrane proteins, expressed by the recombinants constructed with NS1 and a part of E and NS2a sequence were also transferred to the cell surface. The mice inoculated with the E protein produced by the recombinant Baculovirus or infected with the recombinant Vaccinia virus which expressed it on the cell surface developed neutralizing antibodies and protective activity against JE virus encephalitis.

IDENTIFICATION OF A NEUTRALIZING EPITOPE OF ENVELOPE GLYCOPROTEIN (E) OF DENGUE VIRUS TYPE 2.

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The dengue virus genome is a positive-stranded RNA which encodes a polyprotein of 3391 amino acids in length which is then processed into three structural proteins: capsid (C), membrane (M) and envelope glycoprotein (E) and at least seven non-structural proteins. Many monoclonal antibodies (Mab) which exhibit flavivirus group-specificity, as well as dengue virus type-specificity have been previously characterized in other laboratories, some of which can neutralize the infectivity of dengue virus. One neutralizing dengue type 2-specific monoclonal antibody 3H5 reacts with E glycoprotein which is expressed on the surface of the virus. Here, we describe a novel approach for mapping the epitope recognized by 3H5 Mab. First, E protein was expressed in *Escherichia coli* using an inducible promoter λP_L . The immunoreactivity of this 3H5 Mab was localized within 180 amino acid coding region in the C-terminal half of the protein using a series of in-frame deletion constructs expressing truncated E polypeptides. Second, in order to map the epitope more precisely, a series of well-defined deletions within this region were made using polymerase chain reaction and synthetic oligodeoxynucleotides. By testing each deletion construct for expression in *E. coli* and its reactivity with 3H5 antibody, its epitope was determined to be within a region of 12 amino acids in length. A synthetic peptide consisting of this epitope was shown to react specifically with 3H5 Mab by ELISA. Production of antisera specific to this peptide is in progress.

MOLECULAR BASIS OF VIRAL ANTIGENS: LINKAGE OF SEQUENTIAL AND CONFORMATIONAL NEUTRALIZATION EPITOPES OF POLIOVIRUS, TYPE 1.

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Monoclonal antibodies (mAbs) against sequential neutralization epitopes were obtained by a combined in vivo/in vitro immunization protocol. The antibodies recognized VP1 or VP2 in an immunoblot and could be competed by synthetic peptides representing residues 93-104 of VP1 or 164-170 of VP2, respectively. The epitopes involved at least residues 97-101 of VP1 or residues 165-167 of VP2 as defined by amino acid substitutions of resistant mutants. Cross-neutralization tests of the mutants with mAbs against conformational epitopes revealed a linkage between these sequential and conformational epitopes. Insight into the structure of the conformational epitopes was obtained by mutants, where a point mutation resulted in a substitution in a neighbouring loop of the sequential epitope. Thus, the conformational epitope of VP1 is formed by residues within loop 93-104 and 141-152 at the 5-fold axis of the virion. The conformational epitope of VP2 resides within a double loop structure, formed by residues 127-185 near the 2-fold axis of the virus particle. This combination of antibodies offers valuable tools for the study of virus neutralization or of structural changes during virus morphogenesis and virus-cell interactions.

HETEROGENEITY IN THE HUMORAL IMMUNE RESPONSE TO NS1 DIMER AND NS1 MONOMER IN PATIENTS WITH DENGUE INFECTION.

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Sera obtained from serial bleeds of patients (from Thailand and Malaysia) with dengue fever or dengue haemorrhagic fever were analysed by Western blotting for reactivity to the monomeric and dimeric forms of NS1 glycoprotein of dengue virus. Infected and uninfected cell lysates (C6/36 and PS Clone D) were compared, and the virus used was the dengue type 2 strain 16681.

We have evidence that the major response to virus infected cells in humans naturally infected with dengue virus, is to the NS1 dimer. The antibody response to NS1 monomer is a relatively late response, and in a number of patients we have not been able to detect antibody to NS1 monomer, even in convalescence, although these patients have a strong response to the dimeric form of NS1 from early in the course of the illness.

The subclasses of IgG responsible for the NS1 dimer/monomer specificities have been investigated, and we have evidence that IgG3 may be primarily directed at the NS1 monomer.

These data are compared with data from immune but well donors, and their relevance to pathogenesis of dengue haemorrhagic fever is discussed.

THE ANTIGENIC STRUCTURE OF POLIOVIRUS TYPE 2: SEROTYPE CROSS-REACTIVE MONOCLONAL ANTIBODIES IDENTIFY A NEW SITE

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Previous studies have identified an antigenic site (site 1) common to all three poliovirus serotypes (VP1 residues 89 to 100) and two further complex sites. Site 2 consists of residues 220-222 from VP1 (site 2a) and residues 164-172 from VP2 (site 2b). Site 3 includes residues 286-290 from VP1 (site 3a) with residues 58-60 and others of VP3 (site 3b). Neutralising monoclonal antibodies (MCA) prepared against the Sabin and Lansing strains were used to isolate and characterise neutralisation - resistant mutants of Sabin type 2. Twenty-six MCA selected mutants at the expected frequencies which could be assigned to four distinct groups of reactivity by cross-neutralisation tests. RNA sequencing of antigenic mutants in group 1 revealed single base substitutions corresponding to amino acid changes in site 1. Group 2 antigenic mutants possessed amino acid substitutions in site 2a, which was extended in type 2 poliovirus by a mutation at residue 217 in VP1. Similarly, group 3 mutants collected in site 2b, also extended by a mutation at position 158 in VP2. Mutants in group 4 were selected with five MCA, three of which cross-react between serotypes 1 and 2 in neutralisation and antigen blocking experiments. Nucleotide sequencing has located the antigenic site identified both by group 4 mutants and by mutants of Sabin type 1 selected with cross-reactive MCA. Antigenic site 3 appeared to be common only to serotypes 1 and 3.

ANALYSIS OF THE CELLULAR RECEPTORS FOR BOVINE CORONAVIRUS

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Bovine coronavirus (BCV) has been reported recently to use the same kind of receptors for attachment to erythrocytes as influenza C virus. The evidence is based on the finding that BCV contains the same receptor-destroying enzyme (Vlasak et al., PNAS, 85, 4526-4529, 1988) as influenza C virus the enzyme of which has been identified as sialate 9-O-acetyl esterase (Herrler et al., EMBO J., 4, 1503-1506, 1985).

We have performed resialylation studies with erythrocytes to analyze the receptors for BCV. Only red blood cells containing N-acetyl-9-O-acetylneuraminic acid were found to be agglutinated by BCV while cells containing sialic acid without an acetyl residue at position C-9 were resistant to agglutination. Incubation of MDCK cells with purified acetyl esterase from either BCV or influenza C virus rendered the cells resistant to infection by BCV. This finding indicates that O-acetyl residues are also used by BCV as receptor determinants to initiate an infection.

RGD SYNTHETIC PEPTIDES AFFECT FOOT AND MOUTH DISEASE VIRUS ADSORPTION TO CELLS. Yechiel Becker* and Barry Baxth*, Dept. Molecular Virology, Faculty of Medicine, Hebrew University, Jerusalem, Israel, and * Molecular Biology Laboratory, USDA, ARS, Plum Island Animal Disease Center, Greenport, NY 11944, USA.

The major antigenic domain in the VP1 structural protein of foot and mouth disease virus (FMDV) of serotypes O1 (A12), O1 and C3 contains the amino acid sequence RGD. Isolates of serotype A contain attachment sequence in fibronectin receptor (E. Ruoslahti, Ann. Rev. Biochem. 57:375, 1988). To determine the role of the RGD sequence of FMDV VP1 in the interaction of the virus with cellular receptors, we examined the effect of synthetic peptides RQDS, RQDL and a control peptide RPDS on the binding of radiolabeled FMDV to bovine kidney cells. The synthetic peptides were purified by HPLC before use and incubated with cells for 4 hr prior to the addition of ³⁵S-methionine-labeled FMDV type A12. The peptide RQDL at a concentration of 200 µg/ml inhibited virus adsorption by 60-70 % at the same concentration. The control peptide RPDS inhibited virus adsorption by 25-30 %. The inhibition was dose dependent, but less than that achieved by the use of saturating amounts of unlabeled A12 virus to compete with the binding of the labeled virus. These results are in agreement with a recent report on the role of RQDS sequence in FMDV (Acharya et al., Nature 337:709, 1989). The RQDS and RQDL peptides were able to inhibit the binding of a number of other serotypes A, O or C suggesting that the RGD sequence is important for adsorption of the virus to susceptible cells. Since the FMDV subtype O1K contains RQDS in VP2 and RQDL in VP3, the possibility that FMDV virions attach to more than one receptor molecule needs to be explored. Computer analysis revealed the presence of RG sequences in rhinovirus-2 and 1a VP1 (RGLF), poliovirus VP1 (RGAC) and hepatitis A VP1 (RGL). It is not yet known if these sequences are involved in the attachment of these viruses to receptors.

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THE UPTAKE OF SEMLIKI FOREST VIRUS (SFV): ROLE OF THE CAPSID (C)-PROTEIN.

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SFV infects susceptible cells by the endocytotic pathway (Helenius et al., J. Cell Biol. 84, 404 (1980)). We had performed hydrophobic interaction chromatography to investigate the relevance of viral binding to the fusion event occurring at low pH between the viral and endosomal membranes. We found that: 1) the E protein was responsible for binding; 2) the E protein concomitantly underwent a conformational change upon binding; and 3) low pH treatment of bound virus led to a dramatic increase in the hydrophobicity of the E protein, which may be of importance for the fusion event. We have prepared viral particles, which are devoid of E₂ and E₃, and found that these E₁-viral particles are infectious. This showed that E₁ alone is necessary and sufficient for infection. In addition, we found that binding of the virus to the hydrophobic column also triggered digestion of the C-protein. We suggest that the conformational change of the E₁ occurring upon binding concomitantly transmits a signal across the viral membrane leading to digestion of the C-protein. This would: 1) yield greater freedom for the spikes to move in the lateral plane of the viral membrane and thus facilitate the fusion event occurring in the endosomes; and 2) initiate the unraveling and uncoating of the nucleocapsid necessary for the release of the viral genome after fusion.

ANALYSIS OF FOOT-AND-MOUTH DISEASE VIRUS NEUTRALIZING IDIOTYPES FROM IMMUNE BOVINE AND SWINE WITH ANTI-MURINE IDIOTYPE PROBES.

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Rabbit anti-mouse anti-idiotypic antibodies (aids) induced by foot-and-mouth disease virus (FMDV) neutralizing monoclonal antibodies were used as probes to identify anti-FMDV idiotypes in immune sera from bovine and swine. In a competitive radioimmunoassay, four of the aids exhibited a dose dependent capacity to compete with labeled virus for anti-FMDV antibodies from a convalescent bovine serum. Two of the aids that exhibited the highest activity were immobilized on activated Sepharose, and used to isolate anti-FMDV antibodies from bovine, swine, and murine FMDV immune sera. Both the bovine and swine antibodies recovered from the aid-Sepharose columns reacted with virus, and to a lesser extent, with corresponding monoclonal antibody resistant viral variants. The binding of the bovine and swine anti-FMDV antibodies to virus was specifically inhibited by the homologous aid, and in addition, bovine and swine idiotypes were capable of neutralizing FMDV in both suckling mouse protection and plaque reduction neutralization assays. Therefore, by means of aid probes generated against FMDV murine idiotypes, two neutralizing idiotypes were identified in bovine and swine. These results suggest that FMDV neutralizing epitopes recognized by murine systems can play a role in the overall immunity of FMDV susceptible animals.

THE CYTOTOXIC T CELL RESPONSE TO FLAVIVIRUS

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T cell responses to flavivirus infection may be important as an essential component of a protective immune response; alternatively, they may play a role in immunopathogenic disease such as DHF/DHSS. MHC Class I restricted T cells are thought to recognize epitopes consisting of a peptide derived by processing of endogenously synthesized viral protein which is bound to a groove in the MHC class I molecule; the peptide/MHC combination is expressed on the cell surface. We have generated from 5 mouse strains MHC Class I restricted cytotoxic T cells against the flavivirus Kunjin, and used them to attack a panel of target cells infected with one out of eight recombinant vaccinia viruses, which between them contain cDNA of the entire Kunjin genome. We have found that: 1. Most Kunjin immune cytotoxic T cell responses are directed against epitopes derived from non-structural, cytosolic viral proteins. In most mouse strains, epitopes derived from structural proteins are recognized weakly or not at all. Epitopes from NS1 are not recognized. 2. The strongest epitope(s) are derived from a region of 100 amino acids surrounding the NS3/NS4a cleavage site. 3. Other epitopes recognized by at least one mouse strain include one derived from NS5 or the carboxyl-terminal of NS4B (with H-2^D); one derived from NS4A/NS4B (with H-2^D); and a second epitope in NS3 remote from NS4A (with H-2^D). 4. The influence of MHC on Kunjin epitopes recognized is profound: no one Kunjin sequence is recognized by all mouse strains. For any given Kunjin epitope, most MHC alleles are non-responders: even the "immunodominant" site around NS3/NS4a is recognized with only 3 of the 8 MHC alleles we have been able to test.

CHIMAERIC VIRUSES IDENTIFY NEUTRALISING ANTIBODIES TO POLIOVIRUS ANTIGENIC SITES IN HUMAN AND ANIMAL SERA
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Four distinct antigenic sites have been defined on poliovirus types 1 and 3 which differ in their immunogenicity in mice. This study reports a direct approach to analysis of the neutralising antibody response of humans and other animals to type 3 antigenic sites by using antigenic chimaeras. The chimaeras tested had antigenic site 1 (VP1 89-100) of Sabin 1 replaced with antigenic site 1 of type 3 strains. Standard neutralisation tests were performed with panels of sera with neutralising activity against type 3 but not type 1 virus. Between 25 and 50% of human sera from individuals infected with live virus (wild type or vaccine) neutralised the chimaeras depending on the precise sequence inserted. This shows that antigenic site 1 is an important site for neutralisation of poliovirus type 3 in at least some individuals. Similar evidence was found in rabbits, guinea-pigs, monkeys and mice immunized with live virus. In animals ability to neutralise correlated with strain of immunizing virus. For example sera from guinea-pigs and monkeys immunized with antigenic variants of type 3 did not neutralise the chimaeras. Sera which fail to neutralise site 1 chimaeras may have antibody directed predominantly against other antigenic sites. Chimaeric viruses will be ideal tools to investigate this possibility.

ANTIGENIC AND FUNCTIONAL ANALYSIS OF THE FLAVIVIRUS E-GLYCOPROTEIN USING SYNTHETIC PEPTIDES.

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We have used computer analysis to derive synthetic peptides from the deduced amino acid sequences of the E-glycoproteins of Murray Valley encephalitis (MVE) and dengue 2 (D2) viruses. Peptide immunogenicity appeared to depend on inclusion of a putative helper T-cell epitope as predicted by the Rothbard motif. Three immunogenic regions were identified on both viruses: 1) amino acids 35 to 170, 2) 225 to 275, and 3) 300 to 370. Peptides from all three of these regions elicited antiviral antibody and were readily detected by human infection immune sera. A peptide from amino acids 35 to 50 elicited virus neutralizing antibody. Non-linear, "structural" peptides which included a sequence conserved by all flaviviruses (amino acids 98 to 111) elicited antibody which recognized pH 5 denatured virions better than native virions. This result implicates this conserved region in flavivirus mediated cell fusion. An important helper T-cell epitope was identified in domain 2. Domain 3 corresponded to a region previously defined by lambda expression of glycoprotein fragments. A functional helper T-cell epitope was also defined in this region. Competitive binding assays define significant overlap of all three of these defined domains in the native glycoprotein.

IMMUNOCYTOCHEMICAL STUDIES ON POLIOVIRUS RECEPTORS; ITS TOPOGRAPHY, DISARRAY, AND REMOVAL BY MONOCLONAL ANTIBODIES AND SECOND LIGANDS.

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The cellular receptor for poliovirus shows a cluster-like distribution at the cell surface, as demonstrated with immunocytochemical methods in electron-, light- and laser-scanning microscopy using replica-immunogold- and fluorescence probes. The cluster-like distribution is no longer visible after incubation of unfixed, native HeLa- or Vero-cells with the monoclonal antibodies D170 or D710 preventing poliovirus infection (Nobis et al., J. gen. Virol. 1985, 66, 2563). The antibody-receptor complexes are not internalized by the cells, and no ultrastructural alterations are detectable within the receptor areas, neither in ultrathin sections nor in freeze-fracture preparations. The complexes can still move in the plane of the plasma membrane. Up to 24 hours they can be reaggregated by a second ligand; leading to the formation of large positively labelled immunocomplexes (Mannweiler et al., Inst. Phys. Conf. Ser. No. 93, 311, 1989). These immunocomplexes condense in the perinuclear area and are removed from the cell surface either by extrusion or by internalisation via non-coated endocytic vesicles into membrane-bound compartments of the cell.

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A Monoclonal Antibody Defined Epitope Map of Expressed Rubella Virus Protein Domains

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An expanded library of murine monoclonal antibodies (mAbs) was generated by infecting Balb/C mice (Virology 143:153,85) and selecting secreted hybrids by ELISA using purified virion targets. A panel of cDNA clones encoding the structural proteins of rubella virus (RV) was provided by Dr. T. Frey (Gene 62:85,88). Plasmids containing specified RV cDNA fragments were constructed using a variety of strategies into the pGE374 expression vector. RecA-RV-LacZ trihybrid proteins over-expressed in *E. coli* were then probed with selected mAbs by ELISA and immunoblot approaches. Current data localize mAb defined epitopes within the following domains: Mabs C1, C2, C8 bind epitopes within the predicted amino terminal 29 amino acids of the capsid region C1-C29; mAbs E2-1 through E2-6 bind to the E2 glycoprotein backbone region E21-E2115; mAbs E1-18 and E1-20 bind to the E1 glycoprotein region E162-E1273. All of these mAbs appear to react with "linear" epitopes. MAb E1-18 neutralizes RV infectivity, mAb E1-20 neutralizes infectivity and blocks hemagglutination. A synthetic peptide (SP2) representing C14-C29 induced polyvalent antibody reactive to SP2, the appropriate expressed trihybrid protein and RV in ELISA. These and other plasmid constructions and SP will be presented with a strategy useful in deducing the molecular organization of antigen sites of this human pathogen.

MOLECULAR ANALYSIS OF THE ANTIGENIC STRUCTURE OF FOOT-AND-MOUTH DISEASE VIRUS (FMDV)

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The knowledge of the FMDV antigenic structure is necessary for the development of recombinant and synthetic vaccines against viral infections. For this purpose neutralizing monoclonal antibodies (Mabs) were prepared against FMDV strain O_{Kaufbeuren} and synthetic peptides. The monoclonal antibodies were characterized by various immunological methods including Elisa, Western Blot and plaque reduction assays and could be divided in six different groups. The antibody binding sites were localized by competition experiments and amino acid sequence comparison of nine different FMDV strains tested in a plaque reduction assay.

In addition Mab resistant mutants were selected and the protein sequence of their complete P1-coat protein region were determined by c-DNA sequencing. Regarding these results we have identified at least 6 different epitopes involved in virus neutralisation. Two of them are conformation dependent, the others represent sequential epitopes.

LOCATION OF NEUTRALIZATION DETERMINANTS IN THE E PROTEIN OF MURRAY VALLEY ENCEPHALITIS VIRUS. R.C. Weir*, Eva Lee, Suzanne Hartley, J.T. Roehrig and L. Dalgarno. Biochemistry Dept., Faculty of Science, The Australian Natl. University, Canberra, Australia.

The locations of antigenic determinants involved in the neutralization of the flavivirus, Murray Valley encephalitis virus, have been defined in terms of their positions in the amino acid sequence of the E protein. Two approaches were used. Firstly, monoclonal antibodies (mAbs) specific for the E-1c epitope were used to select neutralization resistant variants. Nucleotide sequencing of the envelope protein genes (E and M) of the variants showed that in each variant there was a single nucleotide change in the E gene indicative of a non-conservative amino acid substitution in the E protein at position 126 or 128. Secondly, expression of segments of the E protein as *β*-galactosidase fusion proteins in *E. coli* has allowed the location of the binding site for another epitope specific mAb (E-8) to the amino acid sequence 200-223. These results are in accord with the molecular model proposed for the antigenic structure of the flavivirus E protein (C.W. Mandi *et al.* J. Virol. 63: 546-571, 1989).

ISOLATION OF DENGUE VIRUS (DEN) TYPES 2 AND 3 FROM LEUKOCYTES OF PATIENTS HOSPITALIZED WITH DEN INFECTION. BL Innis, A Nisalak*, S Nimmannitya. Dept of Virology, AFRIMS and Children's Hospital, Bangkok, Thailand.

Over 5 months in 1988, we isolated DEN (2 DEN-1, 13 DEN-2, 13 DEN-3) from 28/75 children with confirmed infection whose plasma or washed peripheral blood mononuclear leukocytes (PBLs) were separately cultured in *Toxorhynchites* mosquitoes. DEN was recovered from both plasma and PBLs in 20 cases, from PBLs alone in 6 cases and from plasma alone in 2 cases. Virus isolation from PBLs appeared to be equally frequent in primary compared to secondary infections (3/8 vs 23/67), despite the failure to recover any DEN-2 viruses from primary cases, and in dengue fever compared to hemorrhagic fever (DHF) (5/13 vs 21/61), even when cases were stratified by the presence or absence of anti-DEN IgM. 13/25 cases with no anti-DEN IgM in the isolation specimen had positive PBL cultures. 5/6 cases where DEN was isolated from PBLs alone had detectable IgM (i.e. the specimen was obtained relatively late in the infection). Geometric mean minimum infection rates (MIR) of PBLs were higher in cases bled early (IgM negative) vs late (IgM positive) ($p=0.043$). MIRs appeared to be higher for primary infections compared to secondary infections. Differences between MIRs of DEN-2 and DEN-3 infections were not observed. Early in infection, many DEN patients have virus replicating in PBLs. Enhanced replication may not occur among those with symptomatic secondary DEN-3 infections, nor among patients with DEN-2 or DEN-3 DHF compared to those with dengue fever. Target cells other than PBLs or host or virus factors other than heterotypic antibody may be critical in the pathogenesis of DHF.

THE SITES OF MULTIPLICATION OF VIRULENT AND DEMYELINATING SEMLIKI FOREST VIRUS IN THE MOUSE CENTRAL NERVOUS SYSTEM

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The virulent L10 strain of Semliki Forest virus causes a lethal encephalitis in the central nervous system (CNS) of weanling mice following intraperitoneal infection, whereas the M9 mutant derived from it is avirulent but induces CNS demyelination. In SJL, but not BALB/c mice, M9 induces persistent demyelination in the absence of virus persistence. We have used *in situ* hybridization with a riboprobe, and immunogold-silver staining with polyclonal rabbit anti-SFV in the CNS of both BALB/c and SJL mice. In both mouse strains at 3-5 days post infection, L10 infects both neurons and glial cells. For M9, infection of neurons is less intense, but infection of glial cells triggers immune-mediated demyelination.

INDUCTION OF NEURAL TUBE DEFECTS IN THE FOETAL MOUSE FOLLOWING INFECTION OF THE MOTHER WITH THE TS 22 MUTANT OF SEMLIKI FOREST VIRUS

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The A7 strain of Semliki Forest virus (SFV) is avirulent for adult mice when given intraperitoneally, but is rapidly lethal for the developing foetus following infection of the mother. The ts22 mutant derived from A7 is teratogenic for a proportion of foetuses following infection of the mother at day 8 or 10 of pregnancy. We have shown using *in situ* hybridization with nucleic acid probes, and by immuno-gold silver staining with anti-SFV antiserum that skeletal and skin defects are induced by infection of mesenchymal cells of the developing dermis and surrounding cartilage-agenous plate. In contrast to other tissues, the central nervous system was poorly stained by ICSS and *in situ* hybridization. Open neural tube defects were induced indirectly by infection of mesenchymal cells adjacent to the developing neural tube, rather than by direct infection of neuroepithelial cells.

PERSISTENT ENTEROVIRUS INFECTION OF MUSCLE IS ASSOCIATED WITH A DEFECT IN VIRUS GENOME REPLICATION.

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Despite the failure to detect infectious virus or virus-specific antigens in clinical samples, persistent enterovirus infection has been implicated in chronic diseases. This has arisen principally from molecular hybridisation studies using an enterovirus-specific cDNA probe, and more recently, cRNA probes, which have revealed the presence of viral RNA in the affected tissue of a significant proportion of patients with chronic diseases of heart and skeletal muscle.

During productive infections of cells cultured *in vitro*, virus replication involves the asymmetric synthesis of a preponderance of positive strand RNA, via production of negative strand RNA and replication intermediates.

We have shown, by the use of ³²P-labelled riboprobes in slot blot hybridizations, that in Coxsackie B2 virus infected cell monolayers, positive strand RNA is present in greater than 50-fold excess over the negative strand. By Northern blot analysis, this negative strand RNA is present only as a species approx 15 kb in size, probably formed by a hairpin loop to a molecule of the positive strand RNA. However, the majority of the positive strand RNA is present as a 7.4 kb species.

In contrast, tissue samples from patients with chronic muscle diseases due to enterovirus persistence contain approx equimolar amounts of the positive and negative strands of virus RNA.

These data suggest that a defect in enterovirus replication is involved in the establishment of persistent infection *in vivo*.

ATTENUATION OF FLAVIVIRUSES BY PASSAGE IN HELA CELLS

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Several workers have shown that six passages of the wild type yellow fever virus (YF) strain Asibi in HeLa cells, results in the generation of a virus which fails to cause viscerotropic disease in monkeys.

We have examined the effect of six HeLa cell passages on other members of the Flaviviridae. Both mosquito and tick-borne viruses were passaged in HeLa cells and their pathogenicities determined in outbred mice. Three isolates of the mosquito borne West Nile virus (WN) were attenuated following six HeLa cell passages. WN strain Sarawak was attenuated 4,000 fold for mice whereas WN strains Egypt 101 and Smithburn viruses were totally avirulent for mice and had in addition lost the ability to infect monkey kidney cells. HeLa passaged WN-Sarawak differed biologically from the parent virus by monoclonal antibody studies and also the presence of a temperature sensitivity marker. Attenuation of WN-Sarawak in HeLa cells was shown to specifically involve the envelope protein gene. In contrast to the above, the two tick-borne isolates remained fully virulent for mice following HeLa cell passage. The results show that HeLa cell attenuation is not limited to YF strain Asibi, but may however be specific for mosquito borne viruses following six passages. Attenuation of WN virus is correlated with biological alterations in the virus. We propose the use of HeLa cell attenuation in the study of determinants involved in the attenuation of Flaviviruses.

RETROSPECTIVE SEROLOGICAL CONTROL OF PERSONS PREVIOUSLY VACCINATED AGAINST TICK-BORNE ENCEPHALITIS

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Killed Tick-Borne Encephalitis vaccine FSME-Immun prepared by the firm "IMMUNO" in Austria has been used in Hungary since 1977. The seroconversion rate was found to be about 0.75 according to a preceding field trial between 1977 and 1979 (Molnár, E., Erdős, L., Fornosi, F., in: Tick-Borne Encephalitis /Ed. Kunz, Ch./ Baden, Vienna, 1979). No TBE cases has been observed till now among people vaccinated during that period. In contrast to this 14 serologically verified acute TBE cases have been recognized among persons who had been vaccinated with the complete three doses of a further purified vaccine between 1979 and 1983.

The presence of specific TBE antibodies was tested in 1987-88 on 529 serum samples from persons who had received three doses of vaccine between 1977 and 1985. Only 0.58 part of persons vaccinated between 1977 and 1979 remained seropositive. However, the rate of seropositivity was as low as 0.25 among people vaccinated between 1980 and 1983. The overall seropositivity rate was 0.33 in the total group, while in a group of 60 individuals with comparable vaccination history a seropositivity rate of 0.72 was measured several years after a fourth dose of the vaccine. These results indicate the necessity of both the revaccination and the control of postvaccination immuneresponses.

DENQUE VIRAL ANTIGEN(S), ANTIBODIES AGAINST DENQUE POLYPEPTIDES, AND COMPLEMENT C3 IN SERA OF PRIMARY DENQUE INFECTION.

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The findings in sera collected from dengue hemorrhagic fever (DHF) patients during admission, before any treatment had been given, can be summarized as follows:

1. C3 depletion accompanying by the presence of C3 split products correlated with severity of illness.
2. Viruses can be isolated from 9 out of 29 primary dengue infected patients (31.0%). 7 of them were identified as dengue type 3 virus, and the other two were dengue type 1 and type 2.
3. In acute phase, only IgM antibodies to dengue viruses can be detected by ELISA technique. These antibodies are against NS3 and NS5 polypeptides as detected by immunoblot with enzyme immunoassay.
4. ELISA to detect specific dengue IgG antibody and immunoblot to detect IgG antibody to dengue peptide in acute sera revealed negative results in all cases. IgG antibodies to envelope protein (E) and NS3 are the first two antibodies to polypeptide which can be identified in convalescent sera. In some cases, antibody to NS3 appeared before the IgG antibody to envelope protein.

Data suggested that in primary dengue infection, shock syndrome occurred without an existence of IgG antibodies to dengue virus, while IgM antibodies appeared in most cases. Antibodies to the NS3 and NS5, which are the virus nonstructural proteins, appeared rapidly. An existence of dengue viruses, IgM and IgG antibody, C3 split products, and antibodies to dengue polypeptides in connection with the severity of this disease will be presented and discussed.

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IN SITU HYBRIDIZATION ANALYSIS OF TYPE 1 POLIOVIRUS MULTIPLICATION IN THE CENTRAL NERVOUS SYSTEM OF MONKEYS.

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In order to detect virus-containing neural cells in poliovirus-infected monkeys, we developed *in situ* hybridization for viral RNA with a poliovirus-specific riboprobe (nucleotides 221-670 from the 5' non-coding region of type 1 poliovirus (PV-1) Mahoney). Using this method, the presence of poliovirus genomes was studied in histological preparations of the central nervous system (CNS) of monkeys inoculated intraspinally with virulent or attenuated (Sabin) PV-1. In monkeys paralyzed after the inoculation of a neurovirulent revertant of PV-1 (Sabin strain, viral RNA was detected in motor neurons and their processes and in polynuclear and small neural cells. By quantitative *in situ* hybridization, the viral replication at the single-cell level was analysed and it was thus shown that the death of motoneurons was due to the direct effect of poliovirus replication in these cells. The role of polynuclear cells seems to be confined to the removal of necrotic neurons. The study of neural histological lesions of monkeys paralysed after infection with attenuated PV-1 as compared to those infected with the virulent strain revealed two major differences: i) the number of destroyed motoneurons was reduced and limited to the inoculation site and ii) the inflammatory reaction was localized but was more intense.

POINT MUTATIONS AND A LARGE DELETION RESPONSIBLE FOR THE ATTENUATED PHENOTYPE IN NEUTRALIZATION ESCAPE MUTANTS FROM A CHIMERIC TYPE 1/TYPE 2 POLIOVIRUS.

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A chimeric poliovirus was constructed by substituting the antigenic site 1 (amino acid sequence 94 through 102 of capsid polypeptide VP1) of type 1 poliovirus (PV-1) Mahoney strain with that of PV-2 Lansing strain (Martin *et al.*, EMBO J., 1988, 2, 2839). As compared to its PV-1 parent, the antigenic hybrid virus thus obtained (v310) was neutralized by antibodies directed against antigenic site 1 of PV-2 and was neurovirulent for mice. Neutralization escape mutants were isolated from v310 with two PV-2 site 1 neutralizing monoclonal antibodies (H2Bn, H2C (9 mutants) and H2D (6 mutants)). The resistance to neutralization with H2Bn and H2D was not always accompanied by resistance to antibody H2C, confirming the possible independent variation of the two PV-2-specific epitopes. Mutations causing resistance to H2Bn/H2D clustered in the C-terminal part of the VP-2 sequence of VP1 (Lys 99 and Arg 100). While those to H2C were found in the N-terminal of antigenic site 1 at Asp 93 and Asp 95, and also outside the antigenic replacement loop at Leu 104. In one of the escape H2C mutants (516 H1), the whole substituted sequence (aa 94-102) was deleted. As suggested by thermolability of the virus at 45°C, mutations Asp 95 → Gly (but not Asp 95 → Asn), Lys 99 → Glu, Arg 100 → His and a 94-102 deletion altered the capsid stability of the mutated viruses. Like the parental v310, most of the mutants have a good replicative capacity at supra-optimal (40°C) temperature, as the parental v310, except the Asp 95 → Gly variant and the 94-102 deletion mutant, which were ts⁺. While the ts⁺ character was accompanied by the loss of neurovirulence in mice, the ts⁻ marker was not always correlated with virulence, since in 12 of 13 variants the mutation induced an attenuated phenotype.

A COMPARATIVE STUDY ON THE SENSITIVITY OF JAPANESE ENCEPHALITIS VIRUS IN R. MONKEY AND MICE

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The sensitivity of Japanese encephalitis virus (JEV) in monkey and mice is unknown yet. Many researchers on the study of the safety of JE live vaccine should be passed through monkey finally. We have studied the pathogenicity of JEV in monkey and mice and found out that the virulence of JEV was declined following the increasing of passage-times in Primary Hamster kidney cell (PHKC). Concerning the SA14 parent strain, when the LD50 in weanling mice was 5.00-6.33, the LD50 in weanling monkey was also 5.50. When the filial strain was passaged at 50 times, the LD50 in weanling mice was declined to 1.45-2.44, the 4 tested weanling monkeys were sacrificed just only one, its LD50 was <0.0. While it was passaged at 100 times, the LD50 in weanling mice was further dropped to <0.0-3.38, and there were 8 to fall fever, one appearing Neurotic symptoms among the 17 tested weanling monkeys. The 3 strains of live vaccine have completely lost the virulence both in monkey and mice. According to these data, we considered that the pathogenicity of JEV was equal to monkey and mice, even the mice was more sensitive than the monkey. However, the clinical study was not enough to prove using mice in stead of monkey. For this purpose, we have to study the changes of cytopathology caused by JEV both in monkey and mice.

CHARACTERIZATION OF THE YELLOW FEVER VIRUS 17DD STRAIN.
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The Oswaldo Cruz Foundation produces several million doses of yellow fever vaccine each year, accounting for a large proportion of the YF vaccine prepared worldwide. As part of a broader program to determine the molecular basis for attenuation of flaviviruses using YF as a model, the YF 17DD strain should be characterized in detail. We report here on the purification of the 17DD virus directly from virusinfected chick embryo homogenates, which is the source of virus used for vaccination of millions of people in Brazil and other countries for almost 50 years.

Neutralization and haemagglutination tests showed that the purified virus is similar to the original stock. Furthermore, radioimmunoprecipitation of ^{35}S -methionine-labeled viral proteins using mouse hyperimmune ascitic fluid revealed identical patterns for the purified 17DD virus and the 17D-204 stock. Finally, comparison by northern blot hybridizations of virion RNAs of purified 17DD with two other strains of 17D virus (17D-204 and 17D-213) shows genome-sized molecules for all three viruses. We are now constructing a cDNA library of the purified 17DD virus in order to derive the nucleotide sequence corresponding to the vaccine phenotype.

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A SINGLE AMINO ACID EXCHANGE IN THE E-PROTEIN OF TICK-BORNE ENCEPHALITIS-VIRUS LEADS TO ATTENUATION OF MOUSE VIRULENCE

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Variants of tick-borne encephalitis (TBE) virus were selected by growing the virus in the presence of neutralizing monoclonal antibodies. Seven mutants were thus obtained, which differed by the wild-type by only a single amino acid exchange and had lost their capacity to bind the selecting monoclonal antibody.

The amino acid exchanges were located at different sites in the envelope protein E of TBE-virus, corresponding to different antigenic domains.

These mutants were compared with respect to their virulence upon i.c. inoculation of suckling mice and s.c. inoculation of adult mice. One of the mutants revealed a strongly reduced pathogenicity after peripheral inoculation, but retained its capacity to replicate in mice and to induce a high titered antibody response. Infection with the attenuated mutant resulted in resistance to challenge with virulent virus.

EFFECT OF SUBSTITUENT ON THE MODE OF ACTION OF ANTIRHINOVIRAL 9-BENZYL PURINES.

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Rhinoviruses are an economically important and therefore attractive target for chemotherapy. The 9-Benzyl purines are new agents which inhibit replication by binding to the virus capsid. Previous studies have demonstrated similarity in the mechanism of action of BW 429U and 4',6-dichloroflavan (BW683C), a known capsid binding compound. In this study the mechanism of action of two 9-benzyl purines has been compared. BW 429U and BW C160U differ by CF₃ and Cl, at C2 of the purine nucleus. Mutants which are resistant to the compounds are cross-resistant. In potency and yield reduction the compounds are similar but their effects in time of addition and direct inactivation studies reveal that both compounds bind but BW 429U inactivates also. The difference in action may be due to the strongly electron-withdrawing CF₃ substituent. The rhinoviruses have antigenically distinct immunogenic areas on the capsid. This variation in protein may cause the differences in binding affinity of antiviral agents. 9-Benzyl purines may be useful tools in the determination of rhinovirus capsid structure and function.

These studies highlight the difficulties in developing broad spectrum antirhinoviral compounds.

COMPLEMENT AND CIRCULATING IMMUNE COMPLEXES IN THE PATHOGENESIS OF SHOCK AND LEAKAGE IN DENGUE HAEMORRHAGIC FEVER

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Complement system is known to be activated in Dengue Haemorrhagic fever (DHF). Circulating immune complexes (CIC) had been incriminated as the main cause of its activation. To confirm the above findings, complement C3, C4 and anaphylatoxins (C3a and C5a) were measured in the plasma of 36 patients with DHF. Circulating immune complexes were assayed by Clq and Conglutinin binding assays. Soluble terminal complement complex (SC5b-9) was assayed by an ELISA method. Levels of C3 and C4 were found to be low during the period of shock and/or during the subsidence of fever. Their levels correlated inversely with the disease severity. Sharp and high peaks of C3a, C5a and SC5b-9 were detected at the time of the appearance of shock. Small amount of CIC were detected by the two assays and their levels correlated poorly with disease severity.

The above findings confirm the active role of complement/anaphylatoxins in the pathogenesis of shock in DHF; but fail to identify significant amount of CIC. Compared with the profiles of complement activation in typical circulating immune complex disease such as SLE, relatively larger amount of anaphylatoxins were generated in DHF. The complement system was activated to completion in DHF as indicated by the raised levels of SC5b-9 complex. The data indicate a very efficient complement activation in DHF.

THE MECHANISM OF ALTERED SINDBIS VIRUS NEUROVIRULENCE ASSOCIATED WITH A SINGLE AMINO ACID CHANGE IN THE E2 GLYCOPROTEIN

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Changes in the amino acid sequences of both the E1 and E2 glycoproteins of Sindbis virus have been demonstrated to affect virulence. However, the mechanism by which these changes alter virulence is unknown. We have studied two recombinant viruses, TE and TES, containing either Gly (TE) or Arg (TES) at residue 172 in the E2 glycoprotein. TE causes earlier death (3.6 d) than TES (4.8 d, $p < .001$) after s.c. inoculation of newborn mice. Growth of the 2 strains is similar in BHK and L cells but TE grows more rapidly than TES in N-18 neuroblastoma cells and in the brains of newborn mice. TE induces viral RNA synthesis in N-18 cells earlier than TES and binding of TE to N-18 cells is more efficient than binding of TES. These data suggest that the amino acid occupying position 172 of the E2 glycoprotein is important for binding of Sindbis virus to neural cells and that amino acid changes at this position affect neurovirulence.

MODIFICATION OF POLIOVIRION PROTEINS BY HOST PROTEASES
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Selective cleavage of the BC loop of VP1 is a known effect of the intestinal protease trypsin on several poliovirus strains. We have found that plasmin, the activation product of the plasma proenzyme plasminogen, is also able to bring about VP1 cleavage and concomitant antigenic changes similar to those produced by trypsin (Ref.1).

Studies with neutralizing monoclonal antibodies with known target sites in the virion indicated that, as a consequence of the cleavage of the BC loop of VP1, other antigenic sites become more easily accessible to antibodies (2). This conclusion was based on titer increases determined with RACINA, a new assay specifically developed for measuring neutralizing antibodies to protease-cleaved polioviruses (3).

While representatives of all three serotypes of poliovirus can be cleaved by host serine proteases, a type 3-specific phenomenon was an apparent cleavage-induced enhancement of the uncoating phase of the viral replication cycle (2).

These observations suggest that the host enzyme-dependent modification of poliovirion proteins may have a role in the pathogenesis of poliovirus infection in vivo.

References:

- (1) Roivainen et al., submitted
- (2) Roivainen, submitted
- (3) Hovi and Roivainen: J Clin Microbiol 27:709-12, 1989

PATHOGENICITY AND REPLICATION OF ENCEPHALITIC TOGAVIRUSES IN MOUSE ORGANOTYPIC SPINAL CORD CULTURES.

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The pathogenicity of two encephalitic togaviruses, sindbis virus (SV), an alphavirus and West Nile virus (WNV), a flavivirus, was studied in organotypic cultures of fetal mouse spinal cord slices grown in roller tubes. After about 3 weeks *in vitro*, during which time the cultures became abundantly myelinated, they were infected either by 6×10^4 pfu SV or by 8×10^7 pfu WNV per culture. The viruses caused different patterns of cytopathogenicity: SV induced severe cytotoxicity in all glia cells and neurons with concomitant demyelination within 48 hours. In contrast, WNV, even 4 days after infection caused only mild cytopathic effects mainly to neurons and astrocytes and a slight degree of damage to the myelin sheath. A most remarkable finding was the entrapment of WNV particles in the interperiod lines of the myelin sheaths. Treatment of cultures with mouse alpha and beta interferon prior to their infection with either virus protected the cultures from any viral damage. Long-term exposure of noninfected control organotypic cultures of fetal spinal cord slices to mouse interferons had no significant effect on neuronal and glial differentiation, and myelin formation.

DENGUE INFECTION ELICITS CROSS-REACTIVE ANTIBODIES TO PLASMINOGEN.
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Computer analysis of the predicted amino acid sequence of the dengue envelope glycoprotein (serotype 4, strain 814669) revealed that it contains a sequence of 20 amino acids (residues 101 to 121) with similarity to a 21 amino acid sequence contained in a family of serine proteases which function in blood clotting (plasminogen, prothrombin, Factor X, tissue plasminogen activator, urokinase). The dengue envelope sequence in question is highly conserved among flaviviruses. ELISA using murine hyperimmune ascitic fluids indicated that dengue but not Japanese encephalitis (JE) or St. Louis encephalitis virus antibodies could cross-react with plasminogen and prothrombin. ELISA using peptides confirmed the site-specificity of the cross-reactivity with plasminogen. ELISA using human sera from Thai patients revealed that about 70% of individuals experiencing a secondary dengue infection developed a transient cross-reactive antibody response to plasminogen (IgG class antibody detected in acute but not convalescent sera). In contrast, sera from ten patients infected with JE virus were negative compared to a panel of 17 controls. Cross-reactive antibodies are predicted to bind plasminogen 19 amino acids downstream from the serine active site. Further studies are in progress.

USE OF RECOMBINANT VACCINIA VIRUSES EXPRESSING DENGUE STRUCTURAL PROTEINS FOR IMMUNIZATION OF MICE AND PRIMATES. M. Bray¹*, B.T. Zhao¹, J. Strauss², E. Strauss², K.H. Eckels³, R.M. Chanock¹ and C.J. Lai¹. 1. NIAID, NIH, Bethesda, MD 20892 2. California Institute of Technology, Pasadena, CA 3. Walter Reed Army Institute of Research, Washington, D.C.

Recombinant vaccinia virus containing the capsid (C), pre-membrane (pre-M) and envelope (E) glycoprotein genes of strain 814669 dengue 4 virus produces pre-M and E glycoproteins. Mice immunized with this recombinant were protected against an intracerebral challenge of 100 LD50 of strain H241 dengue 4 virus. Vaccinia recombinants expressing only E also protected mice against encephalitis. Although antibodies to E in sera of immunized mice were low or undetectable, resistance to challenge was induced through transfer of serum, indicating that humoral immunity plays a role in protection. A recombinant which contains the C and pre-M genes produces apparently authentic pre-M. Mice immunized with this recombinant were partially protected, indicating that C and/or pre-M may be recognized as antigens and contribute to immunity. A recombinant which expresses pre-M and E of the S-1 candidate vaccine strain of dengue 2 induced solid protection against challenge with 100 LD50 of New Guinea C strain dengue 2. Mice immunized with the dengue 2 recombinant were cross-protected against dengue 4, but animals immunized with the dengue 4 C-preM-E recombinant showed no resistance to dengue 2. Neither recombinant protected against dengue 1.

Rhesus monkeys were immunized with a recombinant expressing dengue 4 pre-M and E and non-structural protein NS1. The monkeys developed antibodies to NS1, but were not protected against homotypic challenge.

PROTECTION BY BACULOVIRUS EXPRESSED PROTEINS OF JAPANESE ENCEPHALITIS VIRUS. J. McCown, M. Cochran, R. Putnak, R. Feighny, J. Burrous, E. Henchal, C. Hoke*. WRAIR, Washington, DC 20307 and MicroGeneSys, East Haven, CT.

The polyprotein gene of Japanese encephalitis virus and genes coding for the E and NS1 glycoprotein were cloned into baculovirus expression vectors and expressed in *Spodoptera frugiperda* cells. Crude cell lysates were administered to C57 black mice in three doses at 0, 3 and 14 days. Mice were bled on day 21 and challenged with approximately 100 LD50 of Nakayama strain Japanese encephalitis virus. Survival was increased from about 30% in control mice to 70% in E and polyprotein recipients ($p < .005$ for both groups compared to control), but was not increased in NS1 recipients, despite the development of antibody by NS1 recipients. Virus neutralizing antibody was demonstrated in 15/20 polyprotein and 18/20 E glycoprotein recipients, as compared with 0/20 control and 1/20 NS1 recipients ($p < .000001$ for E and polyprotein recipients). Antibody against crude lysates of Japanese encephalitis virus infected cells were found in E and NS1 recipients and against purified virion in E recipients only. We conclude that baculovirus expressed E glycoprotein stimulates antibody which is both protective and neutralizing and may form the basis for a vaccine suitable for human use. NS1 antibody was neither protective nor neutralizing.

PURIFICATION OF NATIVE AND RECOMBINANT PROTEINS IN THE PRODUCTION OF DENGUE VIRUS VACCINES. RJ Feighny*, MJ Burrous, CJ Lai, JM McCown, CH Hoke. Walter Reed Army Institute of Research, Washington, DC 20307-5100

Purification of both native and recombinant dengue virus proteins has been accomplished by the use of ion-exchange HPLC technology. Silver stained SDS polyacrylamide gels demonstrate that the proteins are greater than 95% pure, showing one band with native protein from dengue-2 and less than five bands with purified recombinant dengue-4 protein. The proteins are reactive with polyclonal sera in western blotting assays. The purified native dengue-2 envelope glycoprotein has been injected in mice eliciting production of antibody to the protein. Maintenance of antigenicity is highly dependent upon the methods used to disrupt the virus or cells. Different detergents have been used, nonidet P-40 is superior for the disruption of native virion proteins while octyl beta-glucoside is best suited for recombinant proteins. Current experiments are underway to determine the feasibility of using either purified native or recombinant proteins in the production of vaccines.

MOLECULAR MECHANISMS INVOLVED IN THE TEMPERATURE-SENSITIVITY AND ATTENUATION OF SABIN 3 POLIOVIRUS. A J Macadam*, J Hogle¹ and P D Minor. NIBSC, South Mimms, Herts. EN6 3QG, UK and ¹Scripps Institute, La Jolla, CA 92037 USA.

A single amino acid change in the capsid proteins of P3/Leon, the progenitor of the Sabin type 3 vaccine strain of poliovirus, renders the virus temperature-sensitive (ts) and also confers an attenuated phenotype. Reversion of the ts phenotype, whether by direct back mutation or by suppressor mutation, results in loss of attenuation. Such a strict correlation between phenotypes is a prerequisite of any *in vitro* marker for attenuation. Since a ts phenotype may be the result of other, non-attenuating mutations we have been investigating the processes affected by temperature as a result of this attenuating mutation (VP391 ser-phe) in the hope that such information may allow us to screen for attenuation *in vitro*.

The positions of VP391 and suppressor mutations in the 3D structure suggests that a phe at VP391 may result in structural thermolability and that suppressors may act by stabilising the native capsid structure. Differential thermolability between Sabin 3 and P3/Leon has not, however, been found. Other structures, involved in cell entry, uncoating or assembly may be affected. One-step growth experiments showed that RNA transfection did not relieve the ts. Thus there appears to be at least one ts step late in infection. The effect of temperature on assembly processes of the vaccine strain is currently being investigated.

BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF APHTHOVIRUS ATTENUATED STRAINS

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To ascertain the nature of the genetic changes associated with virulence in cattle, we analyzed the biochemical and biological differences between aphthovirus attenuated strains and their respective wild-type strains. As expected due to the high variability of RNA viruses and to the multiple passages used to generate these attenuated strains, molecular weight and charge differences between the polypeptides of the attenuated strains and their respective wild-type strains were scattered throughout the entire genome. A feature common to all the attenuated strains analyzed was an alteration in polypeptide 3A. Sequencing of cDNA copies coding for this polypeptide, identified a genomic deletion, of variable length in the different attenuated strains. Analysis of the in vitro markers of attenuation in primary bovine kidney cells (BK) as well as virulence tests in cattle of recombinant viruses, carrying the 5' or 3' halves of either wild-type or attenuated strains, located the major determinants of attenuation in the 3' half of the genome, although, the 5' half could enhance the attenuation effect carried on the 3' half. Moreover, analysis of functional defects in BK cells demonstrated that the attenuated strains have a diminished capacity for viral RNA synthesis, which was dependent on defects in the 3' half of the genome. In addition a decreased efficiency of viral protein synthesis, which depended on defects in the 5' half of the genome could be observed.

PREPARATION OF A PURIFIED, INACTIVATED HEPATITIS A VACCINE

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A purified, inactivated hepatitis A virus (HAV) vaccine was prepared from human diploid MRC-5 cells infected with the HM-175 strain of HAV. The clarified harvest was inactivated with 0.05% formalin and partially purified and concentrated by ultrafiltration. The concentrate was further purified by isopycnic density gradient centrifugation in Renografin-76 (diatrizoate meglumine and diatrizoate sodium). The major peak of HAV antigen contained complete virions and was separated from subviral antigen and host cell protein. After pelleting, the purified HAV was resuspended to a concentration of 1 ug/ml, adsorbed to alum, followed by addition of a preservative. The purified vaccine (FI-2) was compared to an earlier non-purified vaccine (FI-1) shown to be immunogenic for human volunteers. The HAV-specific antigen in the FI-2 vaccine was increased 139 fold while protein concentration was reduced 100 fold. Additionally, the FI-2 vaccine was > 10 fold more potent in mouse assays than the FI-1 vaccine. These findings demonstrate the feasibility of producing a safe, potent, and pure vaccine that can be used to perform human dose-response studies.

ML-17, a live vaccine strain of JEV, is a M-protein mutant ?

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ML-17 is a live vaccine strain of Japanese encephalitis virus (JEV), created by repeated cell culture passages of JaOH0566, a wild type strain isolated from post mortal human brain. (Yoshida et.al, 1981, Biken J., vol 24, p47-67)

The vaccine strain lacks several parental properties, such as, loss of viremia in swine, lack of developing encephalitis by peripheral challenge in mice and poor multiplication in vector mosquitoes.

We are aiming to understand precise mechanism of the attenuation at a molecular level, and determined nucleotide sequences of the genom RNAs (from 5' termini to the ends of E-protein region, so far).

Six amino acid alterations were accumulated in PrM and M-protein region, though no nucleotide change and no amino acid alteration were observed in 5' non-coding and, C and E protein region, respectively. Comparison of nucleotide sequence on non-structural and 3' non-coding region is still going on. However, the accumulation of six sense mutations only in M-protein strongly suggests that the mutations correlate with unique ML-17 phenotypes.

PATHOGENESIS OF LACTATE DEHYDROGENASE-ELEVATING VIRUS INDUCED POLIOMYELITIS: INVOLVEMENT OF AN ENDOGENOUS RETROVIRUS AND MECHANISM OF ANTIBODY MEDIATED PROTECTION FROM DISEASE. John T. Harty*, Christopher H. Contag, Grant W. Anderson, and Peter G.W. Plagemann. Department of Microbiology, Univ. of Minnesota, Minneapolis, Minnesota, U.S.A.

Susceptibility of mice to the paralytic disease associated with acute lactate dehydrogenase-elevating virus (LDV) infection is genetically linked to the presence of multiple copies of endogenous ecotropic retroviral proviruses which are expressed in these mice after birth. Thus, disease is restricted to certain mouse strains, including C58 and AKR. Paralysis in C58 mice correlates with the presence of LDV RNA and antigens in ventral horn motor neurons of the spinal cord. Accumulation of endogenous retroviral RNA in motor neurons, which is inducible by agents which render mice susceptible to disease, correlates with susceptibility of neurons to LDV infection. Substrains of C58 and AKR mice differ with respect to disease susceptibility which may be related to differences in proviral number or integration sites. Polyclonal and monoclonal anti-LDV antibodies can protect C58 mice from neurological disease when present at the time of infection. The mechanism of protection does not involve elimination of LDV; the antibodies specifically prevent infection of target motor neurons without interfering with LDV infection of macrophages or non-neuronal cells in the CNS. Protective monoclonal antibodies have been used to identify two temporally distinct stages in LDV pathogenesis. These two stages can be separated by an intermediate event required for initial neuronal infection, at which time the virus is not accessible to the protective antibodies. This intermediate event may involve axoplasmic transport of LDV through the peripheral nervous system to the CNS.

BIOLOGICAL VARIATION IN RUBELLA VIRUS STRAINS.

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Eight strains of RV, including both wt+ and vaccine strains have been compared for differences in biological properties and tissue tropism. In vivo these strains are known to vary in their association with induction of both arthritis and neurologic complications following acute infection. In VERO cells, the vaccine strain RA27 reproducibly grew to lower titres than other strains at 35°C, and was relatively temperature-sensitive being almost completely inhibited at 39°C. Differences were also found in the electrophoretic mobilities of the sub-species of E1 and E2 glycoproteins produced in VERO cells by each strain, following Western blot analysis. This antigenic variation was also notable by immune peroxidase (IP) staining of infected VERO cells using several polyclonal antisera to different strains of RV, and also monoclonal antibodies to the E1 glycoprotein of the M33 strain. Both quantitative and qualitative differences in the staining patterns were obtained, and only partial cross-reactivity was found between certain strains, particularly RA27 and Therien.

The relative permissiveness of various differentiated human cells to each strain has also been examined including lymphoreticular cells, primary synovial membrane cultures, a chondrocyte cell-line and mixed cultures of astrocytes and oligodendroglia. Viral yields from each cell type were determined and viral antigen production assessed by IP staining and in some cases, SDS-PAGE. The results have indicated that a wide range of human cell types are permissive to RV and that the RA27 and Cendehill strains show the most selectivity in their choice of host cell.

ANTISENSE OLIGONUCLEOTIDE INHIBITION OF ENCEPHALOMYOCARDITIS VIRUS (EMCV) RNA TRANSLATION

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We report the inhibition of encephalomyocarditis virus (EMCV) RNA translation in cell-free rabbit reticulocyte lysates by antisense oligonucleotides (13-17 mers) complementary to (i) the viral 5' non-translated region (NTR), (ii) the AUG start codon and (iii) the coding sequence. Our results demonstrate that the extent of translation inhibition is dependent on the region where the complementary oligonucleotides bind. Noncomplementary and 3' NTR specific oligonucleotides had no effect on translation. A significant degree of translation inhibition was obtained with oligonucleotides complementary to the viral 5' NTR and AUG initiation codon. Digestion of the oligonucleotide:RNA hybrid by RNase H did not significantly increase translation inhibition in the case of 5' NTR and initiator AUG specific oligonucleotides; in contrast, RNase H digestion was necessary for inhibition by the coding specific oligonucleotide. We propose that (i) 5' NTR specific oligonucleotides inhibit translation by affecting the 40S ribosome binding and/or passage to the AUG start codon, (ii) AUG specific oligonucleotides inhibit translation initiation by inhibiting the formation of an active 80S ribosome, and (iii) the coding region specific oligonucleotide does not prevent protein synthesis because the translating 80S ribosome can dislodge the oligonucleotides from the EMCV RNA template.

MONOCLONAL ANTIBODY RESISTANT ESCAPE MUTANTS OF JAPANESE ENCEPHALITIS VIRUS WITH REDUCED MOUSE NEUROINVASIVENESS.

By Cecilia Dayaraj and E A Gould* NERC Institute of Virology, Mansfield Road, Oxford OX1 3SR.

A panel of monoclonal antibodies (Mab) was prepared using a strain of Japanese encephalitis (JE) virus isolated in Sarawak. The Mab were characterised antigenically by indirect immunofluorescence, plaque reduction neutralisation *in vitro* enhancement of infection and mouse protection tests.

Molecular specifications were analysed by immunoblotting and radioimmunoprecipitation.

Mab that neutralised JE virus were used to derive neutralisation resistant escape mutants from the plaque purified parent strain. The mutants were analysed by immunofluorescence, immunoblotting, haemagglutination and neutralisation tests with the entire panel of Mab, to map the epitopes. The mutants were compared with the parent strain for mouse neurovirulence by intraperitoneal inoculation. Reduced neuroinvasiveness, as judged by decreased virulence was observed with a few escape mutants. The implications of these findings will be discussed.

MOLECULAR DETERMINANTS OF JAPANESE ENCEPHALITIS VIRUS VIRULENCE

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Japanese encephalitis (JE) virus belongs to the family Flaviviridae and is a major cause of encephalitis in humans. The live-attenuated SA-14-14-2 vaccine strain has been characterized by comparison of nucleotide and deduced amino acid sequence with the virulent SA-14. There are 67 nucleotide differences between the two viruses scattered throughout the 10976 nucleotides in the genome. These sequence changes have resulted in 27 amino acid differences in a total of 3432 amino acids. Comparison of the amino acid sequence of the SA-14-14-2 vaccine strain with 3 virulent JE strains: SA-14, JaOArS982, and Beijing 1, reveals that 14 of the 27 changes in the SA-14-14-2 vaccine are unique; 1 in the capsid, 5 in the envelope, 1 in ns2a, 1 in ns2b, 3 in NS3, 2 in ns4a, and 1 in NS5. A transversion U to A occurred in position 39 of the 5'-noncoding region in SA-14-14-2 vaccine strain. Of three nucleotide changes in 3'-noncoding region, 2 are vaccine-specific. Production and genetic manipulation of infectious clones will help to define the specific nucleotide changes which affect virulence.

EXPRESSION OF A GLYCOPROTEIN INVOLVED IN POLIOVIRUS ATTACHMENT CORRELATES WITH THE ORGAN TROPISM OF POLIOVIRUS.

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Unique receptor sites for poliovirus are the primary determinant of the virus' cell and tissue type specificity. An antibody which specifically blocks the cytopathic effects, and binding, of poliovirus was previously generated using HeLa cells as immunogen (Shepley, et al. 1988, PNAS 85:7743). This antibody (designated AF3) detected a 100-kDa protein, upon immunoblot, in only those cell lines and tissues permissive for poliovirus infection. AF3 identified the 100-kDa protein in membrane preparations from human spinal cord, but not in organ homogenates of human kidney or of any murine tissue examined. Furthermore, immunoblot analysis demonstrated an association between expression of the AF3 epitope with human chromosome 19, which is known to confer permissivity to poliovirus infection. A direct correspondence between expression of the 100-kDa glycoprotein and *in vivo* permissivity to infection could be established. In immunohistochemical studies AF3 stained neurons in the reticular formation and clusters of brainstem neurons, consistent with the known pattern of poliovirus damage. In the central nervous system, the antibody appeared to react with synapses. AF3 also reacted with the neuromuscular junction, and not with adjacent muscle. These results are consistent with suspected routes of entry and dissemination of virus. AF3 also bound human T and B cells, but not red blood cells, consistent with the observed replication of poliovirus in Peyer's patches and tonsils. These results strongly suggest that the 100-kDa band detected by antibody AF3 is, or is closely associated with, the poliovirus receptor site. Biochemical purification, and amino acid sequencing of the 100-kDa molecule are in progress.

ULTRASTRUCTURE OF ISOLATED TOBACCO PROTOPLASTS IN THE COURSE OF LONG-TERM INFECTION
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Some of virus-specific abnormalities observed in infected plant cells (virus crystals, X-bodies etc.) are absent in virus inoculated protoplasts. These structures seem to be connected with later stages of infection process. In order to verify this assumption the ultrastructure changes of tobacco protoplasts isolated from preliminary (24 hr) TMV OM inoculated plants were studied for 160 hr after inoculation in sterile conditions of protoplasts incubation. Just after isolation (40 hr postinfection) protoplasts contained virus particles aggregates (VPA) specific to cells of diseased plants. During 24 hr of protoplasts incubation, the VPA were disintegrated and virus particles were spread in cytoplasm, although the virus content per protoplast did not reduced. The pronounced swelling of protoplasts during incubation period was the possible cause for VPA disintegration. X-bodies were absent in protoplasts just after isolation, but small accumulations of osmophilic granules were found. These osmophilic granules were developed into granular structures described us for the protoplasts inoculated in vitro. Later on granular inclusions increased in number and size and became similar to granular-fibrillar inclusions and X-bodies of TMV infected plant cells. Thus the ultrastructure abnormalities specific to virus infected plant cells can be observed in virus infected protoplasts.

AGGREGATION AND DEGRADATION OF TMV PARTICLES IN THE INTERCELLULAR SPACES OF TOBACCO LEAVES.
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Institute of Biology & Pedology USSR Academy of Sciences, 690022, Vladivostok, U.S.S.R.
The fate of tobacco mosaic virus (TMV) particles was studied following their injection into the intercellular spaces of tobacco (*Nicotiana tabacum* L.) leaves of systemic (var. Samsun) and local lesion (var. Xanthi nc.) hosts, as well as leaves revealed systemic acquired resistance (SAR) of plants var. Xanthi nc. As revealed by electron microscopy, an aggregation of infiltrated virus particles takes place immediately after injection. At the same time, the infectivity of the homogenates of infiltrated leaves was found to reduce. In 24-48 h after injection, the leaf homogenate infectivity and the specific infectivity of the TMV preparations isolated from samples was reduced. The drop in infectivity was followed by shorting of TMV virions thus testifying to TMV particles inactivation. No substantial distinctions displayed in the degree of aggregation, degradation and inactivation of virus particles in the leaves of systemic and local lesion hosts. The investigated processes in SAR leaves was found to be enhanced. These results indicated that the mechanisms of the initial defence directed towards pathogene penetration into plant cells are similar in these hosts.

MANIFESTATION OF THE N GENE IN ISOLATED TOBACCO PROTOPLASTS AND CELLS.

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The effect of infection with tobacco mosaic virus on the viability of protoplasts and cells isolated from the leaf mesophyll of *Nicotiana tabacum* L. var. Samsun (a sensitive host) and var. Xanthi nc. (a hypersensitive host) was examined. The leaves were infected before protoplasts and cells isolation, or the protoplasts were infected with virus immediately after isolation. The protoplasts and cells isolated from diseased Xanthi nc. plants during emergence of necrosis were of low viability, but when the protoplasts were infected after isolation from healthy plants they remained viable. No decrease in viability was observed in the protoplasts and cells isolated from the leaves of healthy or diseased Samsun plants, as well as from leaves of healthy Xanthi nc. plants or from the leaves with completed lesions. The N gene was not induced if the infected Xanthi nc. plants were kept at 32°C, the protoplasts prepared from these plants were not destroyed. However, we observed some deterioration of cells isolated from these plants during the early periods of incubation. These results indicated that the tissue integrity, the existence of cell walls in particular, plays a role in the N gene induction and expression.

ULTRASTRUCTURE OF ISOLATED TOBACCO PROTOPLASTS IN THE COURSE OF LONG-TERM INFECTION
Yudakova Z.S., Tanashkina T.V., Zhuravlev Y.N.
Inst. Biol. Pedol., 960022, Vladivostok, USSR

Some of virus-specific abnormalities observed in infected plant cells (virus crystals, X-bodies etc.) are absent in virus inoculated protoplasts. These structures seem to be connected with later stages of infection process. In order to verify this assumption the ultrastructure changes of tobacco protoplasts isolated from preliminary (24 hr) TMV OM inoculated plants were studied for 160 hr after inoculation in sterile conditions of protoplasts incubation. Just after isolation (40 hr postinfection) protoplasts contained virus particles aggregates (VPA) specific to cells of diseased plants. During 24 hr of protoplasts incubation, the VPA were disintegrated and virus particles were spread in cytoplasm, although the virus content per protoplast did not reduced. The pronounced swelling of protoplasts during incubation period was the possible cause for VPA disintegration. X-bodies were absent in protoplasts just after isolation, but small accumulations of osmophilic granules were found. These osmophilic granules were developed into granular structures described us for the protoplasts inoculated in vitro. Later on granular inclusions increased in number and size and became similar to granular-fibrillar inclusions and X-bodies of TMV infected plant cells. Thus the ultrastructure abnormalities specific to virus infected plant cells can be observed in virus infected protoplasts.

METHOD FOR IDENTIFICATION SOYBEAN SEEDS INFECTED BY SOYBEAN MOSAIC VIRUS.
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Various all regions where soybean is cultivated are affected by different viruses, the highest damage resulting from soybean mosaic virus (SMV). Infected plants produce less beans with fewer seeds, the grains being often pigmented to complicate oil refining. SMV is transmitted from generation to generation via seeds, and during vegetation spreads to neighbouring plants the medium of vector-insects. Hence, the use of inoculations of healthy seeds (removal of infection sources) could be effective in combatting viruses. To achieve this aim, the method for obtaining virus-free seeds is necessary.

The luminescence spectra of soybean grains has two maxima. One of this is in ultraviolet, and second is in visible range of spectrum. Ratio of visible luminescence intensity to ultraviolet one for healthy seeds is more than that of virus-infected seeds. This property may serve as criteria for identifying the healthy seeds. A schematic diagram was developed for an installation designed to automatically select of healthy seeds. Ninety-eight per cent of the selected seed material will grow to yield an average of 20 per cent a year. The method is patentable.

TMV STRAIN-SPECIFIC INCLUSIONS IN THE INFECTED TOBACCO PROTOPLASTS
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The ultrathin sections of isolated tobacco protoplasts infected by thermoresistant (tr) and thermosensitive (ts) TMV strains were examined in electron microscope. The virus particles and the virus-specific inclusions (VSI) - granular structures were found in cytoplasm of the protoplasts in 24 hr after inoculation by tr strains (vulgare, OM, nuclear, kazakhstan) and incubation at 24° or 32°. Special type of VSI, the dense osmophilic bodies side by side the virus particles were observed in the protoplasts infected by ts mutant NIIIS at 24°. The virus particles were absent while the number of dense osmophilic bodies considerably increased in the protoplasts at 32°. The capsid protein of TMV mutant NIIIS is known to denature losing of solubility at this temperature conditions. Some other TMV strains producing insoluble coat protein (flavum, PM2) were also demonstrated to accumulate similar dense osmophilic bodies in cytoplasm. Based on this observations one can conclude that it is possible to differentiate between the intact and coat protein defective TMV strains by presence of dense osmophilic bodies in ultrathin sections.

RNA REPLICATION COMPLEX FROM DATURA PLANTS INFECTED WITH POTATO VIRUS X
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RNA-dependent RNA polymerase/RdRp/ activity was found in RNA replication complex isolated from datura plants infected with potato virus X/PVX/. The enzyme was associated with membrane fraction sedimenting as 45% sucrose gradient band and can be solubilized using Triton X-100. Membrane-bound RNA-replicase from PVX-infected plants catalyzed in vitro the synthesis of product that under fractionation on Sepharose 2B contained mainly high molecular weight RNA, genomic length ssRNA and minor quantity of low molecular weight RNA. Analysis of the major high molecular weight RNA-product showed that it corresponded by electrophoretic mobility to dsRNA of $M_r 4.0 \times 10^6$. Denaturation and annealing experiments showed that near 60% of the newly synthesized product was hybridized to genomic PVX RNA. Partially purified preparations RdRp from PVX-infected plants when analysed by PAGE native system contained polypeptides of $M_r 120$ and $190kD$, possessing RdRp activity. That polypeptides were absent in similar preparations from mock-inoculated plants.

MECHANISM OF RNA REPLICATION OF POTATO VIRUS X
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In infected plants, besides cellular RNAs, virus-specific double-stranded RNA (replicative form - RF) is synthesized. It contains terminal poly(A)-poly(U) hybrid and extra G at 3'-end of the minus strand. Except for RF, dsRNAs of less length and co-terminal with 3'-end PVX RNA are synthesized in infected cells. In contrast with other positive-sense viruses replicative intermediate (partly dsRNA with single-stranded ends) have not been detected among virus-specific PVX RNA. Pulse-chase experiments revealed that radioactivity accumulated only in RF and during chase radioactivity reduction was not observed. This evidence suggests that synthesis of PVX RNA carries out through formation an intermediate of conservative type which turns into completely double-stranded structure (RF) during phenol deproteinization. Virus-infected cells, besides genomic-size RNA, also display two major RNA 0.9 and 2.1 kb in length and at least four minor ones of 1.4, 1.8, 3.0 and 3.6 kb. All these RNAs are polyadenylated and co-terminal to the 3'-end of the PVX genome RNA. By their length subgenomic RNAs of 2.1, 1.8, 1.4 and 0.9 kb are similar to appropriate double-stranded RNAs. Subgenomic RNA of 0.9 kb was determined to serve as messenger RNA for coat protein synthesis. Functions of other subgenomic RNAs are under investigation.

PROTEIN KINASE ACTIVITY OF CAPSID PROTEIN OF
RED CLOVER MOTTLE COMOVIRUS
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Red clover mottle virus (RCMV) virions have identical capsids composed of two different proteins (mol. wt 39- and 22 kD). The smaller capsid protein undergoes limited proteolysis resulting in the significant (up to ten times) increasing of the specific infectivity of virus preparation. A cyclic-nucleotide independent protein kinase activity has been demonstrated to be associated with highly purified particles of RCMV. The main acceptors of phosphorylation are the smaller capsid protein and products of its limited proteolysis (20-, 18- and 16 kD). Divalent cations are required for activity. Manganese or calcium at pH 7-8 resulted in optimal incorporation of 32 P radio-label into acid-precipitable protein. Since the divalent cations are required, EDTA was found to be strongly inhibitory. The kinase activity was stimulated by the addition of such polyamines as putrescine, spermidine or spermine. We suggest that such modification of the smaller capsid protein of RCMV as limited proteolysis and phosphorylation may play important role in the viral replication cycle.

STUDY OF PROTEIN PHOSPHORYLATION OF PLANT
mRNPs INFECTED WITH POTATO VIRUS X
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There was found protein-kinase activity in the composition of free and membrane-bound polysomal mRNP and free cytoplasmic mRNP infected plants by PVX. mRNP isolated from infected plants revealed the larger amount of phosphorylated proteins as compared with analogous structures isolated from healthy plants.

Thus, the highest level of phosphorylation of proteins 135,45,38kD was observed in free cytoplasmic infromosomes of infected plants; in free polysomal mRNPs -135,56,45,39,32,25-16kD in membrane-bound polysomal mRNP -149,135,83, 66,41,26kD.

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